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Arômes poivrés des vins et rotundone: aspect sensoriel, appréciation par le consommateur, impact des facteurs environnementaux, viticoles, et des techniques de vinification

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RÉSUMÉ

La rotundone est le principal contributeur à l'arôme poivré des vins rouges. Les travaux menés dans le cadre de cette thèse de doctorat ont permis de faire progresser les connaissances sur cette molécule au niveau sensoriel, sur son appréciation par le consommateur et sur les facteurs environnementaux, viticoles et œnologiques impactant ses niveaux de concentration dans les vins. Une corrélation significative, entre l'intensité des notes poivrées perçues à la dégustation et la concentration en rotundone des vins au sein d'un échantillon composé de 21 vins, a été établie. Le profil de consommation des vins riches en rotundone a également été identifié. Le consommateur préférant les vins poivrés s'apparente à un amateur éclairé possédant un budget par bouteille de vin supérieur aux autres consommateurs. Aucun seuil de rejet n'a pu être établi pour la rotundone et le caractère positif de la molécule a été démontré dans la plupart des cas. Les recherches menées sur l'écophysiologie de la rotundone suggèrent que la production de la molécule a lieu dans la baie et peut être influencée par des facteurs abiotiques (quantité d'eau, niveau éclairement) et biotiques (infection par *Erysiphe necator* et *Botrytis cinerea*). La date de récolte, le clone, et quelques pratiques viticoles ont été identifiés comme des leviers possibles pour favoriser l'accumulation de la rotundone dans les vins. Aucune des techniques de vinification et des variables fermentaires testées n'ont permis d'augmenter les niveaux en rotundone des vins en comparaison avec un témoin vinifié traditionnellement. Ceci signifie que les efforts pour maximiser la concentration en rotundone des vins doivent être entrepris dès le vignoble.

Mots-clés : vin, arôme poivré, rotundone, analyse sensorielle, appréciation par le consommateur, facteurs biotiques, facteurs abiotiques, pratiques viticoles, techniques de vinification

ABSTRACT

Rotundone is the main compound responsible for peppery aroma in red wines. Researches carried out in the frame of this PhD thesis have contributed to advance the knowledge on this molecule from a sensory point of view, on its consumer acceptance and on the environmental, viticultural and enological factors affecting its concentration in wines. A significant correlation between the intensity of peppery notes at tasting, and the rotundone concentration in wines within a panel composed of 21 samples, was established. The consumption profile of those who prefer peppery wines was also identified. Consumers who appreciate wines with a peppery sensory profile are generally wine connoisseurs who are willing to pay more for a bottle of wine than the average consumer. No consumer rejection threshold was determined, and in most cases, the positive character of the molecule was demonstrated. Researches carried on rotundone ecophysiology suggest that rotundone production occurs in grape berries and can be affected by abiotic (amount of water, quantity of light) and biotic (infection by *Erysiphe necator* and *Botrytis cinerea*) factors. The date of harvest, the clone and some viticultural practices were identified as possible leverages to enhance rotundone accumulation in wines. None of the studied winemaking techniques and fermentation variables resulted in enhanced rotundone concentrations in comparison with a control treatment vinified traditionally. This means that efforts to maximize rotundone in wines must be undertaken in vineyards.

Key words: wine, peppery aroma, rotundone, sensory analysis, consumer acceptance, biotic factors, abiotic factors, viticultural practices, winemaking techniques

LISTE DES ABBRÉVIATIONS

CRT consumer rejection threshold

Cys3MH précurseur cystéinylé du 3-mercaptohexanol

DH25 pourcentage de degrés heures supérieur à 25°C

DMS sulfure de diméthyle

FPP farnésyl pyrophosphate

FPPS gène associé à la FPP synthétase

GC gas chromatography

GC – GC comprehensive two-dimensional gaz chromatography

G3MH précurseur glutathioné du 3-mercaptohexanol

IBMP 3-isobutyl-2-méthoxypyrazine

IPMP 3-isopropyl-2-méthoxypyrazine

IPT indice de polyphénols totaux

JA acide jasmonique

LogP coefficient de partage octanol/eau

MeJA méthyl jasmonate

MEP 2-C-méthyl-D-érythritol 4-phosphate

MS mass spectrometry

MVA mévalonate

NDVI normalized difference vegetation index

OAV odour activity value

PDMS polydiméthylsiloxane

PES passerillage éclaircissage sur souche

QTL quantitative trait loci

SBSE stir bar sorptive extraction

SIDA stable isotope dilution assay

SIFT-MS selected-ion flow-tube mass spectrometry

SPE solid phase extraction

SPME solid phase microextraction

SQT sesquiterpène

TCA 2,4,6-trichloro-anisole

TDN 1,1,6-triméthyl-1,2-dihydronaphtalène

VvTPS *Vitis vinifera* terpenoid synthase

3MH 3-mercaptohexanol

3MHA acétate de 3-mercapto-hexyle

4MMP 4-mercaptopentan-2-one

$\delta^{13}C$ rapport isotopique $^{13}C/^{12}C$

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Pourquoi cette thèse ?

Né en Bretagne à la fin des années 1970, rien ne me prédestinait à évoluer un jour professionnellement dans la filière vitivinicole, et encore moins à y exercer une activité de recherche. Lors de mon enfance, les vacances passées dans la région bordelaise ne sont certainement pas étrangères à mon engouement initial pour le vin qui est devenu par la suite, comme c'est souvent le cas, une véritable passion.

Mes diplômes d'Ingénieur Agronome (INP-ENSAT) et d'Œnologue (Université Toulouse 3 Paul-Sabatier et INP-ENSAT) en poche, je n'ai juré pendant mes premières années d'activité professionnelle que par la production. Mes pérégrinations m'ont ainsi conduit dans plusieurs vignobles du monde (Australie, Californie, Argentine, Afrique du Sud et Italie). De retour en France et un peu lassé par les vinifications après avoir participé à 6 vendanges en l'espace de 3 ans, j'ai rejoint le pôle Sud-Ouest de l'Institut Français de la Vigne et du Vin (IFV Sud-Ouest) pour occuper le poste de responsable de la communication régionale.

Pendant 4 ans, mes fonctions se sont limitées à la vulgarisation des résultats techniques de mes collègues auprès des vignerons, des techniciens viticoles et des œnologues de la région. Au cours de ces années, je me suis enrichi techniquement sur la plupart des thématiques liées à la vigne et au vin. Cette période a contribué à façonner le chercheur que je suis aujourd'hui, un chercheur généraliste pouvant s'approprier des thématiques liées à l'amont aussi bien qu'à l'aval.

J'ai ensuite développé mes propres travaux de recherche en collaboration avec des partenaires académiques. Ces expérimentations menées notamment sur l'écophysiologie des arômes poivrés ont apporté de nouvelles connaissances sur un terrain alors vierge. Avec la volonté de diffuser largement ses savoirs, j'ai entrepris de les publier dans des revues scientifiques. Cette voie de diffusion étant peu fréquente à l'IFV, c'est de manière autodidacte et non pas sans une certaine douleur, que j'ai accouché fin 2014 de ma première publication. Depuis lors, 9 autres publications en tant que premier auteur, ont suivi.

En janvier 2017, j'ai rejoint l'Ecole d'Ingénieurs de Purpan (INP-PURPAN) pour enseigner la viticulture, l'analyse sensorielle et l'œnologie, et donner une dimension plus fondamentale à mes recherches. Un an après ma prise de fonction, j'ai initié la rédaction de la présente thèse dans l'objectif de valider, sous la forme de l'obtention du grade académique de docteur, les compétences développées en matière de recherche : recherche de financement, rigueur scientifique, conception de protocole, adaptation de méthodologie, collecte de données, analyses, traitements statistiques, structuration et écriture d'un manuscrit en anglais scientifique. La fonction d'Enseignant-Chercheur ne permettant pas de se consacrer à temps plein à la recherche, ce titre de docteur devrait me permettre de maintenir une activité de recherche soutenue en co-encadrant d'autres travaux de thèse.

Ce manuscrit de thèse traite de la rotundone, un composé fascinant identifié en 2008 comme le principal contributeur aux notes poivrées des vins rouges.

Introduction générale

La composante aromatique d'un vin est un élément important dans son appréciation par le consommateur puisque l'examen olfactif intervient juste après l'examen visuel qui est assez souvent peu discriminant. L'évaluation olfactive se décompose habituellement en deux étapes : le premier nez qui consiste à sentir le vin sans aération ; le second nez qui consiste à sentir le vin après avoir imprimé une légère rotation du verre. Cette agitation permet d'augmenter la surface de contact avec l'air et de favoriser la volatilité des composés aromatiques, notamment ceux qualifiés de lourds au poids moléculaire élevé (Sevkan, et al. 2013). Les arômes sont également perçus lors de l'examen gustatif par voie rétronasale puisque les cavités buccale et nasale sont reliées entre-elles. Ainsi, la persistance des arômes en bouche qui constitue l'un des derniers souvenirs sensoriels laissé par le vin après l'avoir recraché, participe à son appréciation globale.

La teneur totale en substances volatiles des aliments se situe entre 10 et 50 mg/kg (Bauer, et al. 2010). Avec plus de 800 composés (Robinson, et al. 2014) voire plus de 1000 selon certains auteurs (IFV 2013), le vin est l'un des aliments les plus riches en molécules volatiles. Une diversité remarquable est également observée pour d'autres denrées alimentaires. Ont ainsi été identifiés plus de 1000 composés volatiles dans la bière et dans la viande rouge cuite (Parker 2012 ; Ledward, et al. 1992), 800 dans le café (Flament et Bessière-Thomas 2002), 500 dans le pain et dans le cacao (Quílez, et al. 2016 ; Caballero, et al. 2015) et environ 400 dans la tomate (Buttery, et al 1987). Cette richesse aujourd'hui pleinement accessible grâce aux techniques instrumentales, notamment le couplage Chromatographie en Phase Gazeuse (GC) et spectrométrie de masse (MS), est favorisée par les fermentations, les procédés de rôtissage, de cuisson et les réactions de Maillard associées.

Tous ces composés volatils n'ont pas nécessairement de propriété odorante et les odorants sont souvent caractérisés par un poids moléculaire modéré, une polarité faible, une bonne solubilité dans l'eau, une pression de vapeur et un caractère lipophile élevés (Meierhenrich, et al. 2005). Ainsi, 30 composés volatiles contribuent aux arômes typiques de la tomate (Buttery et Ling 1993). Le bouquet du vin implique, quant à lui, environ 80 molécules (Dagan 2006). Les odorants du vin proviennent du raisin ou sont synthétisés lors des opérations pré-fermentaires, des fermentations alcoolique et malolactique, et de l'élevage.

La plupart des contributeurs à l'arôme variétal des vins blancs comme les monoterpénols responsable de notes florales dans les variétés dites muscatées (Terrier et Boidron 1972) ou les thiols variétaux aux arômes de fruit de la passion et de pamplemousse, typiques des vins de Sauvignon blanc (Tominaga, et al. 1998), ont été largement étudiés. La connaissance sur les composés contribuant au caractère variétal des vins rouges, et notamment les composés libres directement extraits du raisin sans être libérés à partir d'un précurseur, était limitée jusqu'à récemment aux méthoxypyrazines, des molécules indésirables à l'origine de nuances végétales (Lacey, et al. 1991).

La découverte en 2008 dans un vin australien de Syrah N de la rotundone, un sesquiterpène responsable de notes poivrées (Wood, et al. 2008), est venue apporter une contribution majeure. Malgré son importance sensorielle, cette molécule est restée longtemps inaperçue, indétectée dans le vin et dans les autres denrées alimentaires, y compris dans le poivre.

Entre 2008 et 2011, les connaissances sur ce composé ont très peu progressé car les conditions climatiques particulièrement chaudes et sèches expérimentées en Australie n'ont pas permis l'obtention de niveaux en rotundone suffisamment élevés pour permettre sa quantification analytique. Par ailleurs, peu de laboratoire se sont penchés sur le sujet car aucun standard commercial n'étant disponible, l'adaptation de la méthode de dosage nécessitait la réalisation préalable d'une synthèse organique.

Dans ce contexte, les travaux initiés en 2011 regroupés au sein de ce manuscrit, sont venus apportés une importante contribution à l'étude de ce composé. Ils ont été menés dans le Sud-Ouest de la France, au sein d'un environnement favorable à l'étude de la rotundone par son encépagement diversifié et ses conditions climatiques de type atlantique dégradé. Sur un terrain encore vierge, ils ont permis i) d'étudier certains **aspects sensoriels** associés à la molécule, notamment la relation entre la concentration en rotundone et l'intensité des notes poivrées à la dégustation, et d'évaluer son **appréciation par les consommateurs** ; ii) de connaître l'impact de **facteurs environnementaux et viticoles** sur la concentration des vins en rotundone; et iii) d'appréhender l'incidence des **techniques de vinification et de quelques variables fermentaires** sur les niveaux de rotundone observés dans les vins, dans l'objectif de fournir aux professionnels du secteur des leviers pour piloter le caractère poivré de leurs cuvées.

Dans un premier temps, un état de l'art sur les arômes poivrés et la rotundone sera réalisé. Seront ensuite présentés les résultats des travaux réalisés sur les différents volets décrits ci-dessus. Un dernier chapitre mettra en relation l'ensemble de nos résultats et proposera quelques perspectives de travail.

Chapitre 1

Etat de l’art sur les arômes poivrés et la rotundone

1. Les principaux odorants du vin

Les **terpènes** figurent parmi les composés les plus représentés dans le règne végétal. Linéaires ou cycliques, ils sont les principaux constituants des huiles essentielles. Seuls les **monoterpènes** et les **sesquiterpènes (SQT),** constitués respectivement de deux et de trois isoprènes (C_5H_8), sont susceptibles d'être odorants. Les **monoterpénols** (linalol, nérol, géraniol, β-citronellol et α-terpinéol) présentant une seule fonction alcool et développant des notes florales, sont des marqueurs typiques des cépages muscatés (Terrier et Boidron 1972). Ces composés peuvent être présents sous forme libre ou glycosylée, liée à un sucre. Le 1,8-Cineole ou eucalyptol est un monoterpène conférant au vin des notes mentholées dont l'origine peut être endogène, ou exogène notamment par transfert aérien à partir de feuilles d'eucalyptus (Capone, et al. 2012). Restés longtemps peu étudiés, les SQT ont connu récemment un regain d'intérêt suite à la découverte de la rotundone dans un vin de Syrah N australien (Wood, et al. 2008). Les SQT font partie de la grande famille des phytoalexines, molécules produites par les végétaux en réponse à un stress.

Les **2-alkyl-3-méthoxypyrazines**, représentées principalement par la 2-méthoxy-3-isobutylpyrazine (IBMP) et la 2-méthoxy-3-isopropylpyrazine (IPMP), sont des hétérocycles diazotés disubstitués. Elles sont responsables de notes végétales (poivron, petit pois, asperge) dans les vins blancs et rouges (Allen, et al. 1991).

Les **composés dits en C6**, principalement sous forme aldéhydique, hexénal et hexanal, sont d'autres molécules responsables de nuances végétales et d'herbe coupée dans les vins. Ces composés sont formés, dès les opérations préfermentaires, par oxydation enzymatique de précurseurs lipidiques (Cordonnier et Bayonove 1981). Lors de la fermentation alcoolique, ils sont majoritairement réduits par la levure en alcool, ce qui limite leur contribution sensorielle dans le vin fini.

Les **phénols volatils** sont d'autres composés volatils contribuant à l'arôme des vins. Le 4-vinyl-phénol, 4-éthyl-phénol, 4-vinyl-gaïacol et 4-éthyl-gaïacol sont les principaux représentants de cette famille chimique. Ils sont formés par décarboxylation, sous l'action de la levure lors de la fermentation alcoolique, des acides phénols cinnamiques (Chatonnet, et al. 1993). Les teneurs en ces deux composés sont en général supérieures dans les vins blancs car la carboxylase est inhibée par les tannins catéchiques des vins rouges. Lorsqu'ils sont présents en fortes concentrations, les dérivés vinyliques sont susceptibles de masquer le fruité des vins, voire d'être à l'origine de défauts dits phénolés. Ces notes phénolées davantage imputables aux éthyl-phénols, sont particulièrement marquées dans les vins contaminés par *Bretanomyces*/*Dekkera* (Fugelsang et al. 1993). Contrairement aux levures de vinification, ces levures possèdent l'équipement enzymatique pour réduire les dérivés vinyliques en dérivés éthyliques.

Les **C13-norisoprénoïdes** constituent une grande famille de composés identifiés principalement sous forme glycosylée. L'aglycone, souvent inodore et libéré par hydrolyse chimique ou enzymatique, subit des remaniements chimiques au pH du vin pour conduire à des molécules odorantes (Sefton, et al. 1993). Ces composés sont formés par dégradation des caroténoïdes, pigments photosynthétiques accessoires de la baie de raisin (Baumes, et al. 2002). La β-damascénone, puissant exhausteur du fruité (Pineau, et al. 2007), le 1,1,6-triméthyl-1,2-dihydronaphtalène ou TDN responsable des notes de kérozène caractéristiques des vieux Riesling (Simpson et Miller 1983) figurent parmi les plus notables représentants de cette famille.

Parmi les composés soufrés contribuant à l'arôme des vins, les **thiols variétaux** ont certainement été les plus étudiés à travers le monde depuis deux décennies et leur découverte dans des vins de Sauvignon (Tominaga, et al. 1998). Le 3-mercaptohexan-1-ol (3MH), l'acétate de 3-mercaptohexyle (3MHA) et la 4-mercapto-4-methylpentan-2-one (4MMP) sont responsables de notes agréables de fruits exotiques, d'agrumes et de buis dans un nombre important de vins de cépage. Ils sont libérés au cours de la fermentation alcoolique par clivage de précurseurs inodores présents dans les raisins et les moûts. Trois voies de synthèse de ces composés ont été identifiés à ce jour : à partir de précurseurs cystéinylés (Cys3MH) (Tominaga, et al. 1998), de précurseurs liés au glutathion (G3MH) (Roland, et al. 2010) et via la voie dite de l'hexénal (Schneider, et al. 2006).

D'autres **composés soufrés légers** moins agréables sont produits lors de la fermentation alcoolique. Il s'agit de l'hydrogène sulfuré, du dioxyde de soufre, du sulfure de carbonyle, du disulfure de carbone, des méthyles et éthylthiols, et des disulfures et thioéthers correspondant. Tous ces composés sont associés aux notes dites de réduction (Rauhut, et al. 1998). Compte tenu de leur volatilité, ces molécules légères sont en général éliminées par le dioxyde de carbone produit lors de la fermentation alcoolique. Des travaux récents ont mis en évidence que la composition des moûts en acides aminés soufrés et en ion métallique, notamment en cuivre, avait un rôle sur la production de ces composés (Franco-Luesma et Ferreira 2016). Le sulfure de diméthyle (DMS) produit au stade fermentaire à partir de la S-méthylméthionine (Loscos, et al. 2008) confère des notes truffées à forte concentration (Segurel, et al. 2004). Lorsqu'il est présent à des niveaux plus modérés, il possède un rôle d'exhausteur du fruité.

Les **alcools supérieurs** et les **acides gras ramifiés** sont des composés fermentaires provenant du métabolisme des acides aminés au cours de la fermentation alcoolique (Bayonove, et al. 1998). Les alcools supérieurs parfois également appelés alcool de fusel, sont représentés par l'isobutanol, l'alcool isoamylique, le cis-3-hexenol et le 2-phényléthanol. Ces molécules volatiles ont une contribution aromatique limitée, plutôt négative puisqu'ils confèrent au vin des notes grossières. Le 2-phényléthanol aux arômes de rose fait exception. Les acides gras ramifiés possèdent une odeur plutôt désagréable mais leur contribution est négligeable aux concentrations retrouvées habituellement dans les vins (Etievant 1991). Les **acides gras linéaires,** également formés au cours de la fermentation par la levure, sont associés au métabolisme des lipides via une voie catabolique et anabolique. La levure ne génère dans les milieux acides que des acides gras à nombre pair d'atomes de carbone, à raison de 10 atomes maximum (Alegre 1982). Les acides gras retrouvés dans le vin sont uniquement les acides acétique, butyrique ou butanoïque, hexanoïque, octanoïque et décanoïque. Geffroy, et al. (2015c) a montré que ces composés participaient aux notes lactées caractéristiques des vins de thermovinification. A partir de ces acides gras, la levure peut générer des **esters éthyliques d'acides gras linéaires**. Elle produit également **des esters éthyliques d'acide gras ramifiés** et des **acétates d'alcool supérieurs** contribuant aux notes fruités des vins blancs et rosés. Il a été montré qu'un moût fortement clarifié, riche en azote et fermenté à basse température favorisait la production d'esters au cours de la fermentation (Moreno, et al. 1988).

Les **lactones** sont des **dérivés furaniques** produites par estérification d'hydro-acides. La 4,5-Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one, plus connue sous le nom de sotolon, et la γ-nonalactone sont les principales lactones susceptibles d'avoir une influence sur l'arôme des vins. Le sotolon, produit au cours de l'élevage, participe aux notes de rancio typiques des vins de Xérès (Martin, et al. 1992). La γ-nonalactone aux notes de pruneaux, a été récemment identifiée comme un marqueur aromatique du vieillissement prématuré des vins rouges (Pons, et al. 2008).

2. L'arôme poivré

2.1 Généralités sur le poivre

2.1.1 Eléments historiques et types de poivre

Originaire de la côte orientale Sud de l'Inde, le poivre noir (***Piper nigrum***) dont les qualités sensorielles sont reconnues depuis des millénaires, est l'une des épices les plus utilisées au monde. Il s'agit de la principale épice importée par les romains. Lors de la tenue du siège de Rome à la fin de l'Empire romain d'Occident, les Wisigoth ont réclamé 3000 livres de poivre en guise de rançon (Bury 1889). Au moyen-âge, le poivre était une denrée très importante dans l'économie européenne puisque les droits de péage se payaient en poivre dans le midi de la France, en Allemagne, en Italie et même en Pologne (Schöll 1830). Cette forte demande a contribué au développement du commerce vers l'Orient. C'est à la recherche de routes alternatives à la longue et périlleuse route des épices, que Vasco de Gamma a doublé le Cap de Bonne Espérance et que Christophe Colomb a découvert l'Amérique (Demolins 1901). Le Vietnam, l'Inde, le Brésil et l'Indonésie sont aujourd'hui les principaux producteurs de poivre à travers le monde.

Les baies vertes récoltées en sous maturité peuvent être conservées en l'état : on parle alors de **poivre vert**. Le **poivre noir**, le plus noble des poivres, est obtenu par fermentation de ces baies vertes. Les baies récoltées à pleine maturité présentent une coloration rouge-orangée et constituent le **poivre rouge**. Le **poivre blanc** est obtenu traditionnellement à partir du poivre rouge, après immersion dans de l'eau pendant plusieurs jours pour éliminer son péricarpe. Le **poivre gris** proposé en poudre, provient du concassage de baies de poivre noir, en général de qualité moindre.

D’autres espèces du genre *Piper* produisent également des poivres. Les baies de *Piper longum* donnent le **poivre long**, celles de *Piper cubeba* le **poivre cubèbe**, et celles de *Piper borbonense* le **poivre de Voatsiperifery**.

Par analogie, d'autres épices reprennent de manière abusive ce nom vernaculaire. Ces baies sont obtenues à partir d’espèces possédant des caractéristiques botaniques différentes et n’appartenant pas au genre *Piper*. Le **poivre du Sichuan** (*Zanthoxylum piperitum*) et le **poivre rose** (*Schinus terebinthifolius*) figurent parmi les représentants les plus connus de ces faux poivres.

En raison de leur **saveur poivrée**, certaines espèces végétales peuvent être qualifiées de poivrées. C’est le cas de la **menthe poivrée** (*Mentha piperita*) issue d'une hybridation spontanée entre la menthe aquatique (*Mentha aquatica*) et la menthe verte (*Mentha spicata*).

2.1.2 Composition chimique et volatile du poivre

La **piperine** et la **chavicine**, son stéréoisomère, sont les principaux composés actifs du poivre. Ces amines secondaires cycliques appartenant à la famille des pipéridines, sont des alcaloïdes responsables de son caractère piquant. Cette sensation repose sur le nerf trijumeau, et non sur les récepteurs chimiques situés dans la cavité buccale responsables des saveurs élémentaires (acide, sucré, amer, salé, umami). Ce nerf assure une fonction motrice pour mordre, mâcher, avaler mais aussi sensorielle puisqu’il est impliqué, en plus du piquant, notamment dans les sensations tactiles, de douleur, de fraîcheur. Au cours du stockage, la chavicine est lentement transformée en piperine ce qui conduit à une baisse du caractère piquant.

Le poivre renferme également de nombreux composés volatiles, majoritairement des **monoterpènes** et des **SQT** (Tableau I). Jagella et Grosch (1999) ont conclu que l’α-pinène, le β-pinène, le myrcène, l’α-phéllandrène, le limonène, le linalol, le 2-méthylpropanal, les 2- et 3-méthylbutanal, les acides butyrique et 3-méthylbutyrique figuraient parmi les principaux odorants de *Piper nigrum*. Il a aussi été démontré que la composition volatile du poivre noir et blanc différait peu (Chen, et al. 2011).

Tableau I : Principaux composés volatiles identifiés dans le poivre (*P. nigrum*)

Monoterpènes	Sesquiterpènes	Autres composés
α-Thujène	α-Copanène	Eugénol
α-Pinène	β-Caryophyllène	Méthyleugénol
Sabinène	β-Bisabolene	Myristicine
β-Pinène	Oxyde de caryophyllène	Safrole
1,8-Cinéole	α-Cis-Bergamotène	Benzaldéhyde
Limonène	α-Trans-Bergamotène	Trans-Anethole
Linalol	β -Bisabolène	Piperonal
Camphène	δ-Cadinène	2-Méthylpropanal
δ^{3}-Carène	γ-Cadinène	2-Méthylbutanal
Myrcène	Calaménène	3-Méthylbutanal
Cis-Ocimène	α-Copaène	m-Méthyl acétophérone
α -Phellandrène	α-Cubénène	p-Méthyl acétophérone
β-Phellandrène	β-Cubénène	n-Butyrophérone
α-Tepinolène	α -Curcumène	Acide phénylacétique
γ-Terpinène	β-Elmènes	Acide cinnamique
Terpinolène	α-Sélinènes	Acide pipéronique
	Rotundone	Acide butyrique
		Acide 3-méthylbutyrique

Adapté d'après Jagella et Grosch (1999), Plessi, et al. (2002), Wood, et al. (2008) et Chen, et al. (2011)

2.2 L'arôme poivré des vins

La dimension épicée a de tout temps été associée à l'univers du vin. Les épices étaient couramment utilisées à l'époque de l'Empire romain pour élaborer le ***conditum paradoxum***. La recette de ce vin épicé est donnée dans l'ouvrage « De Re Coquinaria », écrit au 1er siècle après Jésus Christ par le célèbre gastronome Apicius. Un vin de qualité souvent très médiocre, était bouilli avec du miel puis complémenté avec des épices (poivre, mastic, nard, laurier, safran) et des fruits secs (dattes). Le produit obtenu était ensuite dilué avec du vin de qualité et des charbons ardents étaient ajoutés pour favoriser sa conservation. Cette tradition existait aussi au Moyen-Age puisque l'**'hypocras** désignait un breuvage élaboré à base de vin, de miel, d'épices et d'herbes aromatiques.

De manière plus imagée, la dimension épicée a toujours possédé une place importante dans la description aromatique du vin. Les travaux menés par Noble, et al. (1987) ont permis de classer les arômes du vin en 12 **familles aromatiques** dont une famille épicée (Figure 1). Ces notes épicées (réglisse/anis, poivre noir et clou de girofle) sont clairement dissociables des notes boisées ce qui laisse sous-entendre une origine variétale.

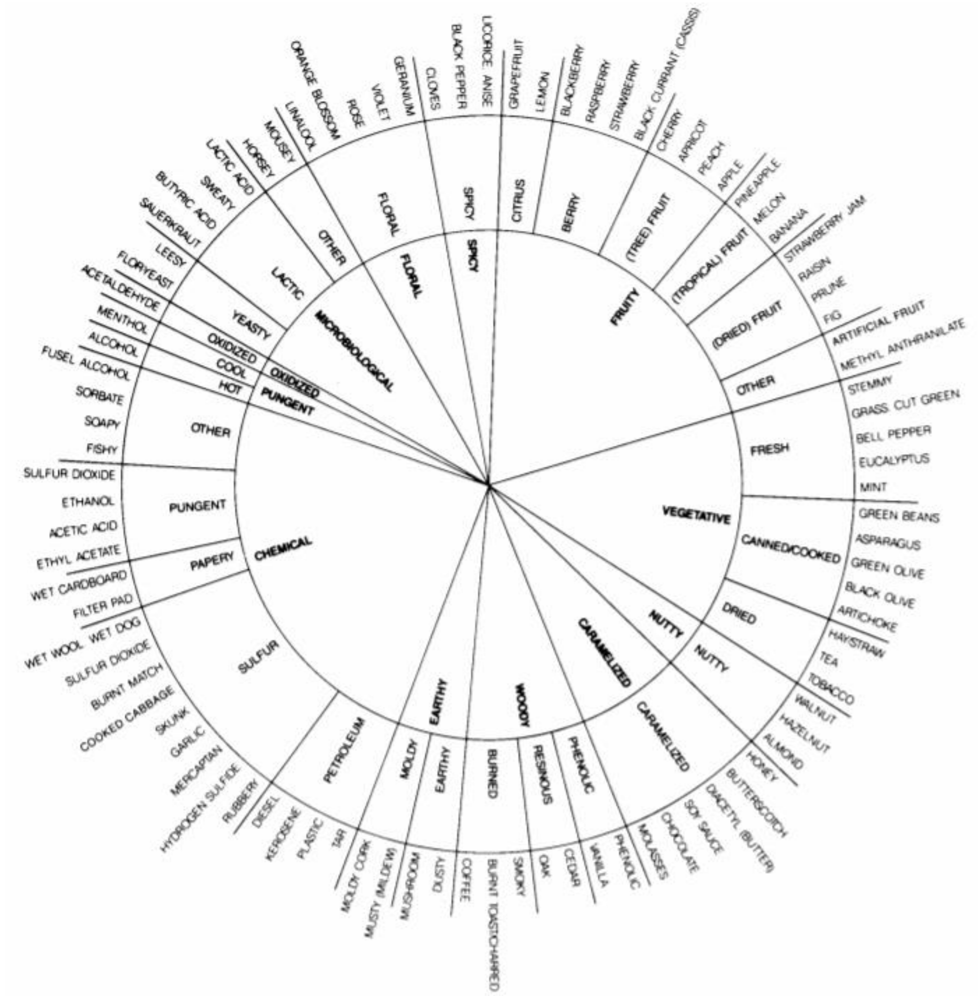

Figure 1 : Roue des arômes proposée par Noble, et al. (1987)

Il est en effet bien connu que certains cépages développent des notes épicées/poivrées caractéristiques. C’est le cas notamment des vins rouges élaborés à partir de cépages internationaux comme la **Syrah N** cultivée dans la vallée du Rhône septentrionale ou dans l’état du Victoria en Australie, ou le **Gamay N** du massif central, de certains crus du Beaujolais ou de Suisse. D’autres cépages régionaux comme le **Duras N**, planté uniquement à Gaillac, la **Mondeuse N** de Savoie ou le **Mouvèdre N** à Bandol présentent également des notes poivrées particulièrement prononcées. De manière empirique, ces notes apparaissent souvent plus marquées lors des millésimes frais ou dans les zones fraiches.

Malgré l’importance de la Syrah N en France (plus de 65 592 ha en 2011 selon le site http://plantgrape.plantnet-project.org/), la recherche française ne s’est que très peu intéressée à l’étude aromatique de ce cépage et aux marqueurs moléculaires associées aux notes poivrées. L’étude la plus marquante réalisée sur notre territoire a été menée sur des vins de Syrah N élaborés dans des vignobles chauds. Elle a mis en évidence que les précurseurs glycosidiques étaient les principaux contributeurs à la typicité aromatique des vins de Syrah N de la vallée du Rhône méridionale (Segurel 2005). Ces composés sont responsables des arômes fruités et d'olive noire. Ces observations sont cohérentes avec d'autres travaux menés 15 ans plus tôt mettant en avant le rôle de ces précurseurs dans les notes terreuses, de tabac/cigare des vins de Syrah N (Abbott, et al. 1991).

La majorité des recherches visant à étudier le **déterminisme moléculaire** des notes poivrées ont été conduites en Australie. Dans le cadre d'un mémoire de spécialisation, Brightman (2000) a étudié par chromatographie en phase gazeuse (*gas chromatography* en anglais ou GC) couplée à de l'olfactométrie et à de la spectrométrie de masse (*mass spectrometry* en anglais ou MS) la composition volatile de grappes de Syrah N. Ces travaux n'ont pas permis d'identifier une seule zone spectrale associée aux notes poivrées/épicées. Quelques années plus tard, Parker, et al. (2007) ont utilisé une approche non ciblée. Des lots de grappes de Syrah N dont l'intensité poivrée a été évaluée par un jury expert, ont été analysés par une méthode associant espace de tête et GC-MS. L'analyse multivariée de 13 000 spectres de masse par grappe a permis d'identifier l'α-ylangène, un SQT, comme le meilleur marqueur/prédicteur de ces notes poivrées dans les raisins. Ces travaux ont également mis en avant que ce marqueur, non retrouvé dans les vins, n'avait pas d'impact aromatique.

C'est seulement en 2008 que la principale molécule responsable des notes poivrées des vins rouges a été formellement identifiée dans un vin australien de Syrah N (Wood, et al. 2008). Il s'agit d'un SQT oxygéné de formule moléculaire $C_{15}H_{22}0$, la (3S,5R,8S)-5-Isopropényl-3,8-diméthyl-3,4,5,6,7,8-hexahydro-1(2H)-azulénone, plus connue sous le nom de **rotundone** (Figure 2). L'un des faits les plus surprenants dans cette découverte, est que ce composé n'avait jamais été détecté dans le poivre malgré le nombre conséquent de publications traitant de la composition volatile de *Piper nigrum* (Jagella et Grosch 1999,Plessi, et al. 2002). Le caractère aromatique distinctif du poivre était jusqu'à alors attribué à l'interaction complexe entre plusieurs odorants (Jagella et Grosch 1999). Le fait que la molécule ait été identifiée aussi tardivement mérite quelques explications. Tout d'abord, la molécule est présente à des niveaux de concentrations de l'ordre du ng/L et est susceptible de co-éluer avec d'autres sesquiterpènes, ce qui rend délicat sa détection. Par ailleurs, la rotundone possède une forte affinité avec la phase stationnaire en GC et un temps de rétention élevé. Elle « ressort » à la fin des séances de *sniffing*, à un moment où l'attention des juges est moindre et où aucune molécule d'intérêt n'est attendue. Finalement, le fait qu'une anosmie spécifique existe pour ce composé a certainement dû compliquer sa détection.

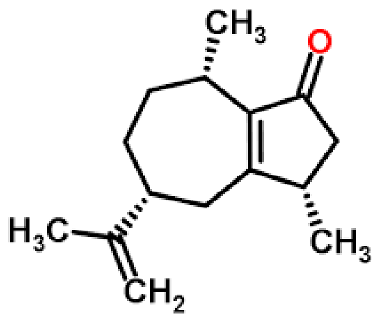

Figure 2 : Structure chimique de la rotundone

3. La rotundone, le principal contributeur à l'arôme poivré des vins rouges

3.1 Quantification de la rotundone dans les raisins et les vins

Plusieurs groupes de recherche à travers le monde ont développé des **méthodes analytiques** de détermination quantitative de la rotundone dans les raisins et les vins. Ces méthodes reposent sur la combinaison **SPE- GC-MS** (Cullere, et al. 2016), **SPE-SPME-GC-MS** (Mattivi, et al. 2011; Nauer, et al. 2018; Siebert, et al. 2008), **SBSE-GC-MS** (Takase, et al. 2015) et **analyse par dilution isotopique**. L'analyse par dilution isotopique ou stable isotope dilution analysis (SIDA) est une méthode de quantification pour laquelle l'étalon interne est un analogue de l'analyte marqué par isotopes stables. Les propriétés physicochimiques de l'étalon et de l'analyte étant très similaires, les biais liés à la préparation et à l'injection sont évités. Dans le cas du dosage de la rotundone, la ***d5*-rotundone**, pour laquelle 5 atomes d'hydrogène ont été remplacés par 5 atomes de deutérium, est utilisée comme étalon interne.

Dans ces méthodes, la préparation des échantillons, indispensable pour augmenter la concentration en rotundone présente à l'état de traces (ng/L), est réalisée par **extraction en phase solide** (*solid-phase extraction* ou SPE), et/ou **micro-extraction sur phase solide** (*solid phase microextraction* ou SPME) ou **extraction par sorption sur barreau magnétique** (*stir bar sorptive extraction* ou SBSE). La **SPE** est une méthode de préparation, au cours de laquelle des composés en solution dans une phase liquide, sont adsorbés sélectivement sur une phase solide, en fonction de leurs propriétés physico-chimiques (Simpson 2000). La phase stationnaire contenue dans une cartouche de la forme d'un corps de seringue, est souvent constituée d'une résine polymérique. Les analytes sont ensuite récupérés par élution, en général à l'aide de dichlorométhane. La **SPME** est une technique d'extraction en phase solide plus récente (Arthur et Pawliszyn 1990). Facile à mettre en œuvre et performante, elle ne nécessite aucun solvant. Cette méthode repose sur l'utilisation d'une fibre en silice ou en verre fondue disposée à l'intérieur d'une aiguille creuse amovible. Une phase stationnaire, en général un film de polymères déterminant la capacité d'extraction, est greffée sur cette fibre. La **SBSE** est une variante de la SPME introduite à la fin des années 1990 (Baltussen, et al. 1999). Le support d'extraction est un barreau magnétique enrobé d'un polymère faisant office de résine absorbante, en général du polydiméthylsiloxane (PDMS). Par rapport à la SPME, cette technique, légèrement plus lourde à mettre en œuvre, permet d'augmenter la quantité de polymère, de diminuer le ratio de phase et ainsi d'augmenter la récupération théorique.

Afin de limiter les phénomènes de co-élution et d'optimiser la séparation, la **chromatographie en phase gazeuse bidimensionnelle** (GC-GC) a également été utilisée pour le dosage de la rotundone (Geffroy, et al. 2014; Takase, et al. 2015). Cette approche consiste à coupler plusieurs colonnes capillaires possédant des propriétés de polarité différentes. La seconde colonne permet de séparer une partie mal résolue du premier chromatogramme et le transfert de la fraction du chromatogramme vers la seconde colonne est dénommée « **coupe à cœur** » ou « *heart cutting* ».

Pour l'**extraction** et la quantification de la rotundone dans les raisins, plusieurs solvants ont été utilisés : l'éthanol (Siebert, et al. 2008), l'acétone (Mattivi, et al. 2011) et le *n*-pentane/acétate d'éthyle (Takase, et al. 2015). Les niveaux maxima retrouvés dans les raisins et les vins finis atteignent 7 µg/kg et 500 ng/L (Caputi, et al. 2011).

3. 2 Occurrence chez les végétaux, dans les vins et les spiritueux

La rotundone doit son nom à l'espèce végétale dans laquelle elle a été découverte : **le souchet d'Asie ou *Cyperus rotundus*** (Kapadia, et al. 1967). La molécule a été détectée dans de nombreuses **espèces aromatiques méditerranéennes** comme le romarin (*Rosmarinus officinalis*), le thym (*Thymus vulgaris*), la marjolaine (*Origanum majorana*), le basilic (*Ocimum basilicum*) et dans *Atriplex cinerea* (Figure 3), un arbrisseau persistant originaire d'Australie (Wood, et al. 2008).

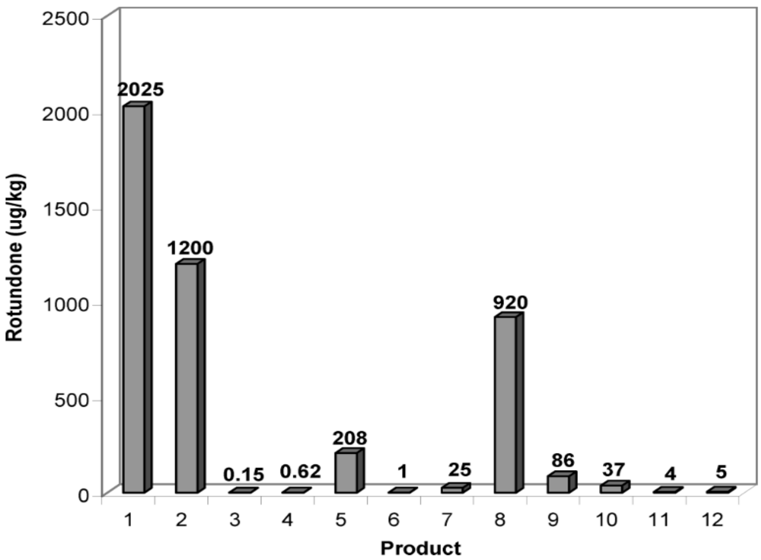

Figure 3 : Concentration en rotundone (µg/kg ou µg/L) retrouvée dans 1, le poivre blanc (*P. nigrum*); 2, le poivre noir (*P. nigrum*); 3, le vin (*Vitis vinifera* cv. Shiraz N); 4, les raisins (*V. vinifera* cv. Shiraz N); 5, la marjolaine (*Origanum majorana*); 6, l'origan (*Origanum vulgare*); 7, le géranium (*Pelargonium alchemilloides*); 8, le souchet d'Asie (*Cyperus rotundus*); 9, le romarin (*Rosmarinus officinalis*); 10, *Atriplex cinerea*; 11, le basilic (*Ocimum basilicum*); 12, le thym (*Thymus vulgaris*) (Wood, et al. 2008)

Plus récemment, il a été montré que la molécule contribuait de façon importante à l'arôme de la **chicorée torréfiée** (Wu et Cadwallader 2019) et de nombreux **jus de fruits** comme ceux de pamplemousse, d'orange, de pomme et de mangue (Nakanishi, et al. 2017a). Dans ces mêmes jus de fruits, un **stéréoisomère** de la molécule, la **3-epi-rotundone**, a également été identifié (Nakanishi, et al.

2017b). Ce nouveau stéréoisomère possède un seuil de perception nettement plus élevé, évalué à 19 100 ng/kg.

La rotundone est une molécule plutôt **ubiquiste** qui a été retrouvée dans de nombreux cépages. Les travaux menés en Australie ont permis de mettre en évidence sa présence à des concentrations dépassant son seuil de perception dans les vins de **Syrah N**, de **Durif N** (la petite Syrah californienne issue du croisement entre la Syrah N et le Peloursin N), de **Mourvèdre N** et de **Graciano N**, un cépage largement planté dans le vignoble espagnol de La Rioja (Figure 4) (Herderich, et al. 2012).

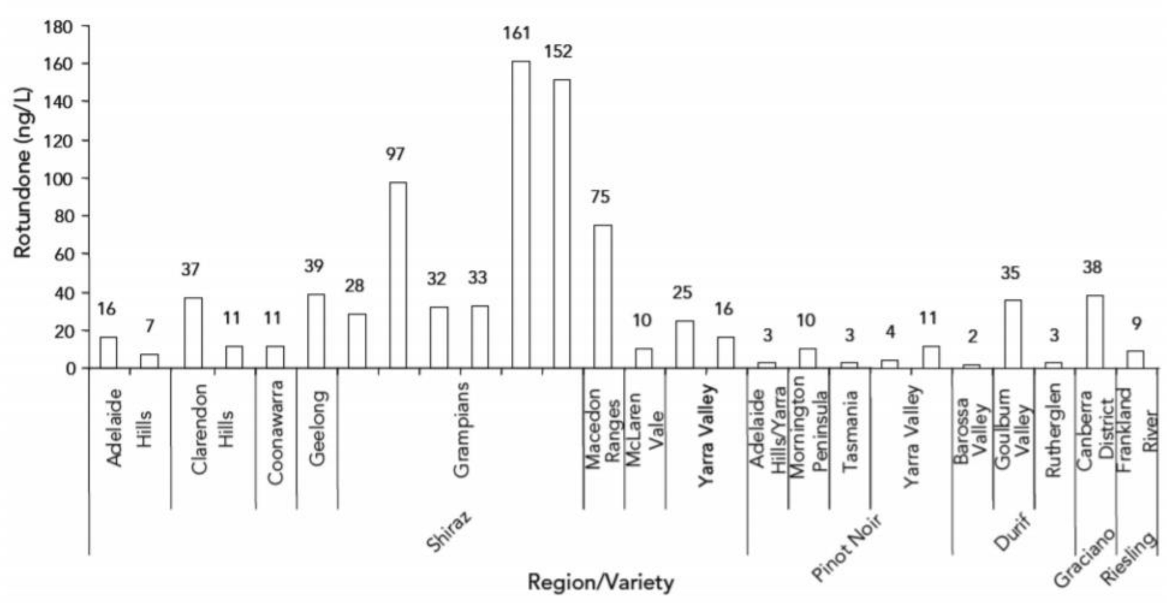

Figure 4 : Concentration en rotundone de quelques vins commerciaux australiens (Herderich, et al. 2012)

La rotundone a également été retrouvée en Italie dans le **Vespolina N**, le **Schioppetino N** et le cépage blanc **Grüner Veltiner B** très répandu en dans le Nord-Est de l'Italie et en Autriche (Caputi, et al. 2011; Nauer, et al. 2018). Plus récemment, elle a été identifiée dans les vins de **Duras N** de **Négrette N**, de **Fer N** et de **Prunelard N** du Sud-Ouest de la France (Geffroy et Descôtes 2017; Geffroy, et al. 2014), de **Gamay N** en provenance de climat frais (Geffroy, et al. 2016a), de **Cot N**, d'**Abouriou N,** de **Castets N** (Cullere, et al. 2016), de **Mondeuse N** et d'**Arani N** (Geffroy et Descôtes 2017). Takase, et al. (2015) ont montré si les raisins de Syrah N produits au Japon possédaient des niveaux remarquables en rotundone, ceux de

Koshu Rs, de Muscat Bailey-A N, de Cabernet Sauvignon N et de Merlot N contenaient des concentrations négligeables.

Le rôle sensoriel de la rotundone a également été démontré dans de **nombreux spiritueux** (bourbon, tequila, rhum, whiskey et brandy) élevés en fûts de chêne (Genthner 2014). Cette étude a permis de mettre en évidence que la concentration en rotundone augmentait avec la durée de vieillissement.

3.3 Aspects sensoriels et appréciation par le consommateur

La rotundone est un composé extrêmement **odorant** puisque son seuil a été établi à 8 ng/L dans l'eau et à 16 ng/L dans le vin rouge (Wood, et al. 2008). Une **anosmie spécifique** a été reportée à ce composé, puisque lors des premiers tests sensoriels, 20 à 25% des membres du panel étaient incapables de détecter la rotundone dans l'eau, même à des concentrations très élevées (> 4 000 ng/L). La molécule a également été décrite comme l'un des 16 composés aromatiques possédant le plus fort **impact sensoriel** dans le vin (Ferreira 2012).

Sur 21 vins de Gamay N en provenance de 4 bassins viticoles français, Geffroy et al. (2016a) ont mis en évidence une **corrélation positive significative** entre l'**intensité des notes poivrées** perçue à la dégustation et la concentration en rotundone (R2 = 0.66). Des résultats comparables ont été obtenus sur cépage Noiret N (Homich, et al. 2017).

Plusieurs études ont été menées afin d'étudier l'**appréciation des notes poivrées** par le consommateur. Williamson, et al. (2012) ont comparé les préférences des consommateurs Chinois et Australiens. Pour les deux marchés, trois groupes distincts de consommateurs ont pu être identifiés. Les principales différences entre les pays concernent les effectifs et la répartition au sein des différents groupes. Alors que deux des trois groupes préfèrent les vins présentant des notes de fruits rouges, douces et fruitées, un troisième groupe apprécie davantage les vins à la couleur soutenue, et aux notes de fruits et de poivre noirs. Dans le cadre d'une étude visant à étudier la typicité poivrée des vins de Gamay de l'AOP Côtes d'Auvergne (Geffroy, et al. 2016a), 4 vins présentant des profils sensoriels différenciés ont été sélectionnés pour participer à une étude consommateur (n = 87). Si aucune différence significative en termes de préférence entre les vins n'a pu être dégagée, une forte opposition a été mise en évidence entre les consommateurs préférant les vins aux profils amylique et poivré. Il ressort également de cette étude que les consommateurs appréciant les vins poivrés sont principalement des **amateurs éclairés** possédant un budget par bouteille supérieur aux autres panélistes. Une autre étude a cherché à établir un **seuil de rejet par le consommateur** pour la rotundone dans un vin de Duras N (Geffroy, et al. 2018), c'est-à-dire une concentration à partir de laquelle la molécule devient indésirable. Aucun seuil n'a pu être identifié et quatre groupes de consommateurs ont été mis en évidence : les anosmiques, un groupe préférant systématiquement le témoin sans ajout, un groupe préférant les niveaux modérés de rotundone et un dernier groupe préférant les vins très riches en rotundone. La réponse des consommateurs vis-à-vis de la rotundone apparaît ainsi complexe.

3.4 Voie de biosynthèse et précurseurs

La **voie de biosynthèse** de la rotundone n'a pas encore été complètement élucidée mais comme d'autres SQT, elle pourrait être synthétisée dans le chloroplaste par la voie du méthylérythritol phosphate (voie non mévalonique ou voie du MEP) et/ou dans le cytoplasme par la **voie du mévalonate** (MVA), en réponse à des attaques d'insectes (May, et al. 2013). Ces deux processus s'excluent mutuellement dans la plupart des organismes mais cohabitent chez les plantes. Chez la vigne, Hampel, et al. (2005) ont montré que si les monoterpènes étaient produits exclusivement dans les feuilles par la **voie du MEP**, la synthèse des sesquiterpènes impliquaient les deux voies et que des exports étaient possibles entre chloroplaste et cytoplasme. Ainsi, le méthyl jasmonate (MeJa) et l'acide jasmonique (JA) ont la capacité à stimuler la synthèse de 25 SQT dans des cellules de baies de Cabernet Sauvignon N et de Riesling B (D'Onofrio, et al. 2009). La régulation de la synthèse des SQT est assurée par les gènes de la famille ***VvTPS*** (Vitis vinifera *terpenoid synthase*), au nombre de 89 dont 45 sont situés sur le chromosome 18 (Martin, et al. 2010). Dans le cadre d'un programme de création variétale visant à développer de nouvelles variétés résistantes aux maladies cryptogamiques de la vigne exprimant des notes poivrées, une équipe de recherche autrichienne a mis en évidence que l'hérédité associée à la production de rotundone était portée par les chromosomes 5 et 9 (Regner, et al. 2016). Pour la rotundone, la voie de biosynthèse ci-dessous a été récemment proposée (Figure 5).

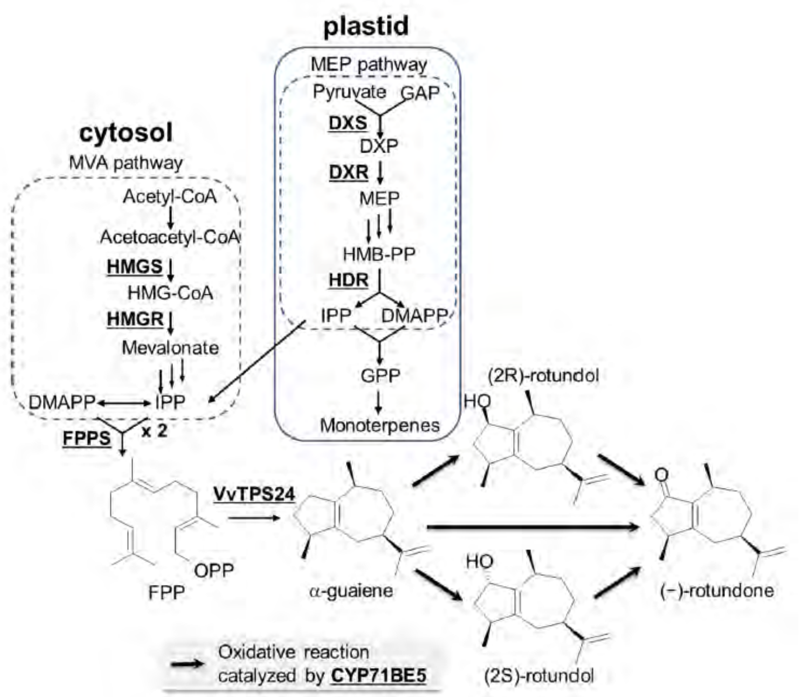

Figure 5 : Voie de biosynthèse proposée pour la rotundone par Takase, et al. (2016)

Le précurseur de la rotundone a été identifié, il s'agit de l'**α-guaiène**. Il existe au moins deux allèles du gène *VvTPS24* chez la vigne et ces deux allèles sont associés à des sesquiterpènes synthétases fonctionnelles (*VvGuaS* et *VvPNSeInt*). L'enzyme *VvGuaS* possède la capacité à transformer le Farnésyl pyrophosphate (FPP) en α-guaiène (Drew, et al. 2016). Ce polymorphisme pourrait permettre d'expliquer la raison pour laquelle la production de rotundone se fait uniquement dans les baies de certains cépages. A partir de l'α-guaiène, la rotundone peut être formée par simple oxydation (Huang, et al. 2014) ou dans le raisin par voie enzymatique (Takase, et al. 2016) grâce à une α-guaiène 2-oxidase de type cytochrome P450 (CYP 71BE5). Les niveaux de transcription de cette enzyme sont plus élevés dans la pellicule des raisins par rapport à la pulpe avec une expression supérieure chez la Syrah N en comparaison avec le Merlot N. D'autres travaux ont mis en évidence que l'expression des gènes *VvTPS24* et *CYP71BE5* ne différait pas entre deux vignobles de Syrah N exposés à des conditions environnementales différentes et possédant des niveaux de production en rotundone très distincts (Takase, et al. 2016). Dans cette étude, le principal élément permettant de discriminer les vignobles était le niveau de transcription du gène associé à la FPP synthétase (*FPPS*). Les mêmes auteurs ont reporté des niveaux d'expression différents pour ce gène entre la Syrah N et le Merlot N. La régulation de la production de rotundone apparaît complexe et sous dépendance des gènes ***VvTPS24, CYP71BE5*** *et* ***FPPS***. Si ces trois gènes sont susceptibles d'expliquer des différences de prédisposition variétale, l'expression du dernier gène pourrait également être régulé par les conditions environnementales.

3.5 Fonction biologique et mécanisme de défense

La **fonction biologique** de la rotundone est toujours inconnue mais comme d'autres sesquiterpènes, la molécule pourrait être impliquée dans les **mécanismes de défense** de la vigne notamment en réponse à des attaques d'insectes (D'Onofrio, et al. 2009). L'application foliaire exogène d'acide jasmonique au vignoble, une phytohormone impliquée dans les mécanismes de défense vis-à-vis des herbivores, n'a pas permis d'augmenter la concentration en rotundone des vins (Geffroy, et al. 2014). Selon cet auteur, il reste délicat de dresser des conclusions définitives, car l'efficacité du traitement est fortement dépendante des conditions d'applications et notamment, de la pénétration de la bouillie au sein de la végétation. De la même manière, l'activité physique (perforation) ou chimique (phytohormones) des **herbivores** sur les feuilles de vigne possède un impact très limité sur la production de rotundone (Zhang, et al. 2016b). Une corrélation positive ($R^2 = 0.58$) a été mise en évidence entre l'intensité des dégâts liés à l'**oïdium** sur grappes et la concentration en rotundone dans les vins élaborés à partir d'une sélection de raisins sains provenant des mêmes grappes (Figure 4). L'oïdium serait ainsi, capable de provoquer une réponse systémique se traduisant par la production de rotundone (Geffroy, et al. 2015a). Selon le même auteur, la différence clonale de sensibilité à l'oïdium pourrait permettre d'expliquer les différences de richesse en rotundone observées parmi les 4 clones agréés de Duras N. La **pourriture grise** et la **pourriture acide** n'induisent pas les mêmes réactions de défense chez la vigne (Geffroy, et al. 2019a; Geffroy, et al. 2016b). Au contraire, dans le cadre d'une étude visant à hiérarchiser les principaux facteurs permettant d'expliquer les différentes concentrations en rotundone au sein d'un réseau de 10 parcelles de Duras N du Gaillacois (Geffroy, et al. 2019a), l'acide gluconique, un métabolite secondaire de *Botrytis cinerea*, a été identifié comme une variable clé négativement corrélée à la rotundone ($R^2 = 0.69$). Ces éléments suggèrent que le champignon est capable de neutraliser les effets toxiques des sesquiterpènes par oxydation vraisemblablement enzymatique ou par détoxification.

3.6 Localisation dans la baie de raisin et dans les organes herbacés

Le décorticage de baies de Syrah N et de Vespolina N a montré que la rotundone était quasiment absente de la pulpe, des pépins et était principalement localisée dans la **pellicule des raisins** (Caputi, et al. 2011; Siebert, et Solomon 2011). Selon Capone, et al. (2012), la concentration en rotundone dans les vins rouges pourrait être influencée par la présence de feuilles et de tiges, organes où la molécule a été identifiée. Des études récentes ont également montré que la molécule était présente dans les **boutons floraux** et que les **tissus herbacés** (pétiole, pédicelle, rameau) contenaient des concentrations en rotundone supérieures à celles mesurées dans les baies (Zhang, et al. 2016a). Cependant, le même auteur a exclu l'hypothèse selon laquelle le composé pouvait être véhiculé depuis ces organes vers la baie par l'intermédiaire du phloème. Geffroy, et al. (2016b) sont arrivés à la même conclusion, puisque le sectionnement de la branche à fruit 18 jours avant la récolte n'a pas stoppé l'accumulation de la rotundone. Cette hypothèse d'une **production *in situ*** est cohérente avec d'autres travaux (Geffroy, et al. 2014) mettant en évidence que la modulation des relations de type source-puit via l'éclaircissage des raisins n'impactait pas significativement les niveaux en rotundone des vins.

3.7 Impact des facteurs environnementaux et viticoles

3.7.1 Variabilité inter-millésime

Tout comme les autres composés aromatiques d'origine variétale, la rotundone est fortement impactée par les caractéristiques climatiques du millésime. Les millésimes froids et humides apparaissent particulièrement favorables à l'obtention de vins riches en rotundone (Caputi, et al. 2011; Zhang, et al. 2015b). La même parcelle de Duras N (AOP Gaillac), suivie entre 2008 et 2015 et récoltée à un laps de temps identique après véraison (Figure 6), montre une amplitude de concentration variant de 7 à 179 ng/L entre les millésimes les plus extrêmes (Geffroy et Descôtes 2017). De la même manière, Bramley, et al. (2017) a reporté, sur une même parcelle, une variation de concentration dans les raisins d'un facteur 40 entre millésimes.

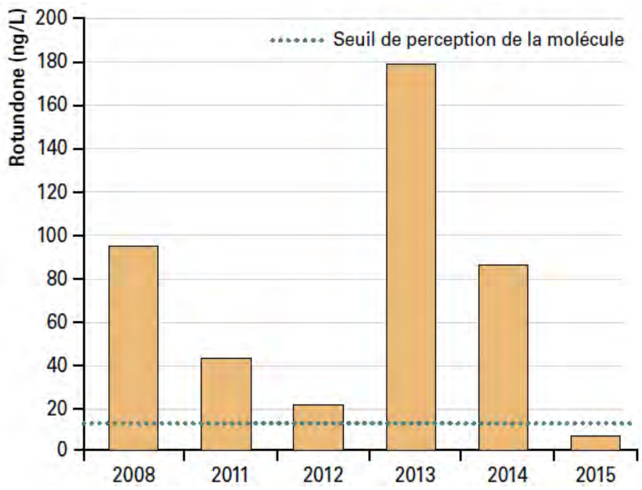

Figure 6 : Impact du millésime sur la concentration en rotundone des vins provenant d'une parcelle de Duras (AOP Gaillac) suivie entre 2008 et 2015 (Geffroy et Descôtes 2017).

3.7.2 Variabilité intra-parcellaire

Des travaux ont mis en évidence que la **distribution de concentration** en rotundone au sein d'une même parcelle était organisée spatialement (Figure 7) et que les motifs structuraux étaient stables d'une année sur l'autre (Bramley, et al. 2017). Les variations observées sont à relier aux **propriétés du sol** et à la **topographie** (Scarlett, et al. 2014), au **microclimat des baies** notamment au pourcentage d'heures au-dessus de 25°C (Zhang, et al. 2015a) ou à l'indice de fraîcheur des nuits (Geffroy, et al. 2019c), et au niveau de **contrainte hydrique** subi par le végétal (Geffroy, et al. 2014). Ces constatations rendent possible l'organisation de récolte sélective dans l'objectif d'élaborer des vins présentant des niveaux distincts de concentration en rotundone et d'expression de notes poivrées. Il a été démontré que la circonférence des troncs, un indicateur du niveau de contrainte hydro-azotée subie par la vigne depuis sa plantation, pouvait être utilisée pour approcher la distribution spatiale de la rotundone (Geffroy, et al. 2015b). Des travaux récents ont essayé de faire le lien entre la **diversité microbienne du sol** et la concentration en rotundone des raisins (Vadakattu, et al. 2019). Les auteurs ont mis en évidence que dans les zones favorables à l'obtention de forts niveaux en rotundone, les sols possédaient une plus grande diversité de bactéries mais une plus faible diversité de champignons.

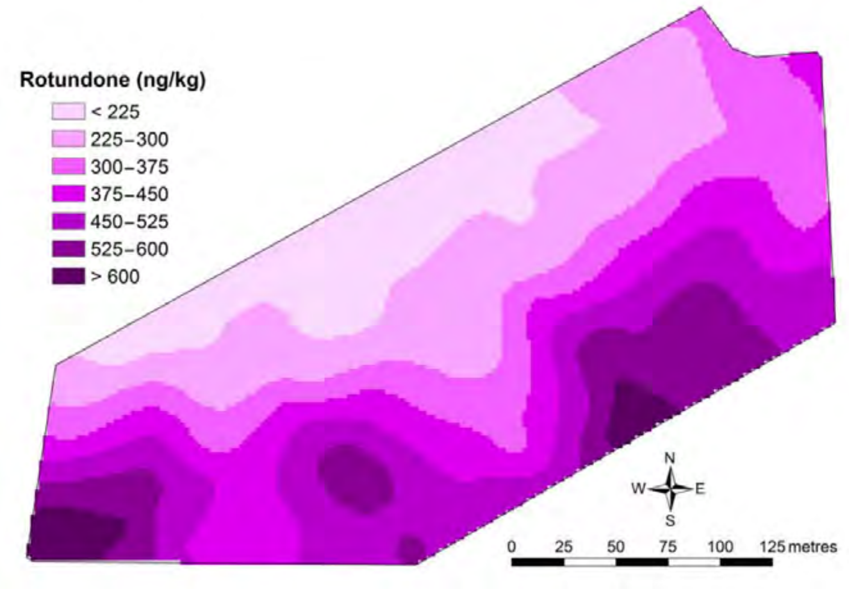

Figure 7 : Variation de concentration des raisins en rotundone sur une parcelle de Syrah de 6.1 ha de la région des Grampians en Australie (Scarlett, et al. 2014)

3.7.3 Matériel végétal

En plus de l'effet cépage décrit précédemment, le **clone** possède un impact sur la richesse des vins en rotundone. En Australie, le clone de Syrah 2626 est réputé pour donner des vins épicés. Il a été démontré qu'il présentait à la récolte des concentrations en rotundone plus élevées que le clone 1127 (Siebert et Solomon 2010). Des variations clonales ont a également été décrites en Italie sur le cépage Grüner Veltliner B (Caputi, et al. 2011). Dans le Sud-Ouest de la France sur cépage Duras N, des concentrations supérieures en rotundone ont été retrouvées dans les vins des clones 554 et 654, en comparaison avec le 555 (Geffroy, et al. 2015a). Le **porte-greffe** par la vigueur conférée au greffon et son impact sur le micro-climat des baies, est susceptible d'impacter la richesse en rotundone des raisins. Des travaux préliminaires menés dans le Sud-Ouest sur 9 porte-greffes ont montré que le Fercal et le 196-17 Castel avaient tendance à produire des vins plus riches en rotundone (Olivier Geffroy, données non publiées 2016).

3.7.4 Maturité et date de récolte

Des résultats contradictoires ont été obtenus quant à la cinétique d'accumulation de la rotundone, de l'inflorescence jusqu'à la récolte. Si la rotundone a été détectée à des concentrations importantes avant véraison dans les **capuchons floraux** sur Syrah N (Zhang, et al. 2016a), elle reste indétectable sur Noiret N au stade fermeture de la grappe (Homich, et al. 2017). Alors que Zhang, et al. (2016a) puis Luo, et al. (2019) ont reporté une cinétique d'accumulation en forme de U (Figure 8), Homich, et al. (2017) ont montré que son accumulation débutait seulement au stade post-véraison. Un consensus existe cependant pour les dernières semaines avant la maturité qui sont caractérisées par une accumulation rapide de la molécule (Caputi, et al. 2011; Geffroy, et al. 2014; Logan 2015; Siebert et Solomon 2010). Dans le Sud-Ouest de la France, les concentrations atteignent un plateau à partir de 44 jours après mi-véraison (Geffroy, et al. 2014). La date de récolte apparaît ainsi comme un levier important pour piloter la concentration des vins en rotundone. Sur cépage Fer N (AOP Marcillac), un décalage de la date de récolte de 7 jours permet un gain en rotundone dans les vins de l'ordre de 120% (Geffroy, et al. 2019b). D'autres travaux ont permis de modéliser la cinétique d'accumulation de la rotundone dans les raisins à partir de la véraison (Zhang, et al. 2015b). Le meilleur modèle a l'allure d'une **sigmoïde** dont les paramètres (pente au point d'inflexion, hauteur du plateau…) dépendent des caractéristiques climatiques du millésime.

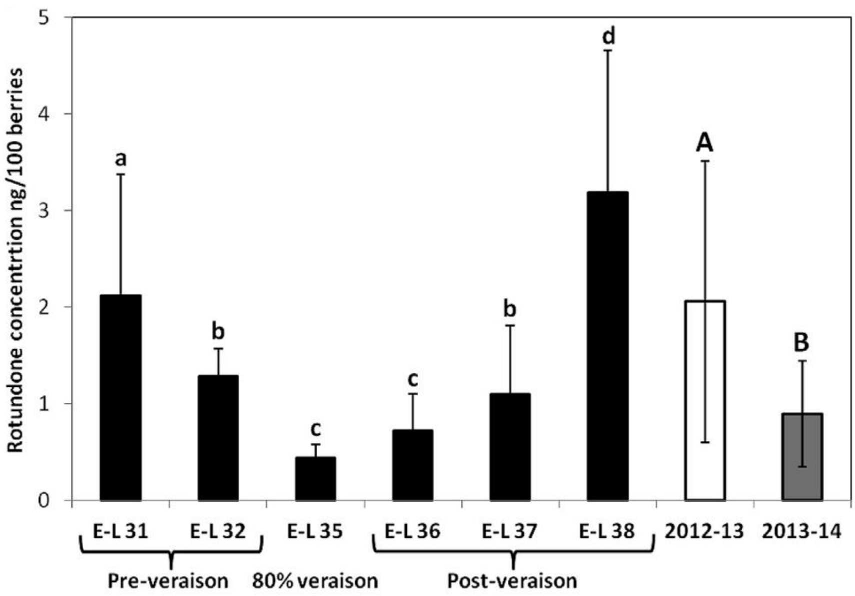

Figure 8 : Comparaison de la concentration des raisins en rotundone (ng/100 baies) à différents stades phénologiques au cours des millésimes 2012-2013 et 2013-2014 (Zhang, et al. 2016a)

3.7.5 Pratiques viticoles

Dans la plupart des cas, la synthèse de la rotundone est affectée négativement par l'**exposition de la zone des grappes**. Un modèle a permis d'établir que des températures de baies supérieures à **25 °C** impactaient négativement la rotundone sur Syrah N (Zhang, et al. 2015a). Sur cépage Duras N dans le Sud-Ouest de la France, un **effeuillage** réalisé à la véraison sur les deux faces du rang pénalise fortement (de -53 à -69%) la concentration en rotundone dans les vins (Geffroy, et al. 2014). En Nouvelle-Zélande, il a également été reporté qu'une augmentation de l'exposition à la lumière réduisait de 20% la concentration en rotundone des baies de Syrah (Logan 2015). Cependant, des résultats contradictoires ont été obtenus dans le Nord-Est des Etats-Unis sur cépage Noiret N puisque qu'une exposition prolongée de la zone des fruits au rayonnement a engendré un gain en rotundone (Homich, et al. 2017). Une tendance similaire a également été observée sur Fer N produit dans un vignoble au climat frais (Geffroy, et al. 2019b). Selon les auteurs de la première étude, la blessure physique provoquée par l'effeuillage pourrait stimuler la production de rotundone. Ceci semble peu plausible puisque l'échardage, une pratique consistant à éliminer les entre-cœurs, n'induit pas le même effet (Geffroy, et al. 2019b). Comme d'autres sesquiterpènes, la synthèse de la rotundone est susceptible d'être favorisée par le rayonnement. Dans les vignobles au climat frais, l'effeuillage pourrait favoriser l'éclairement des raisins tout en ayant un impact limité sur leur température de surface. Cette hypothèse est cohérente avec des observations mettant en évidence une contribution positive de l'**irradiation moyenne** et de l'**ensoleillement** au cours de la période mi-véraison – récolte, pour prédire la concentration en rotundone des vins (Geffroy, et al. 2019a).

Les vins issus d'un traitement irrigué juste avant la véraison (4 apports de 10 mm) présentent une concentration en rotundone de 29 à 38% plus élevée que des vins issus d'une modalité non irriguée (Geffroy, et al. 2014). L'**irrigation** constitue ainsi un levier efficace pour favoriser l'accumulation de la rotundone dans les raisins. Afin d'améliorer la concentration en rotundone des vins, tout en limitant l'effet dépréciatif de l'irrigation sur la richesse phénolique des vins, un système viticole combinant 5 irrigations équivalentes à 14 mm de précipitations et **passerillage éclaircissage sur souche** (PES) a été expérimenté avec succès (Geffroy, et al.

2016b). La technique du PES consiste à sectionner sur une vigne conduite en guyot la branche à fruits, 2 à 3 semaines avant la récolte. Le système testé présente par rapport au témoin, un gain significatif en degré potentiel, en composés phénoliques et en rotundone. Dans le cadre de cette étude, des mesures de température ont montré que l'irrigation n'avait pas d'impact sur la température de surface des raisins. Ainsi, le gain en rotundone induit par l'irrigation est davantage susceptible d'être dû à un effet direct plutôt qu'à un effet indirect, conséquence d'une modification du microclimat des baies.

La concentration en rotundone dans les vins n'est pas impactée par l'**éclaircissage** des grappes et le niveau de charge en raisins (Geffroy, et al. 2014). En Australie, des essais ont été menés afin de retarder la maturité en ayant recours à des pulvérisations d'acide naphtalène acétique à 50 mg/L, une auxine de synthèse (Davies, et al. 2015). En retardant la maturité de 23 jours, cette pratique s'est avérée intéressante pour améliorer la concentration en rotundone des raisins à la récolte.

3.8 Impact des techniques de vinification et des variables œnologiques

Contrairement à d'autres composés aromatiques provenant de précurseurs inodores ou formés pendant la fermentation, la rotundone est directement extraite de la pellicule du raisin pendant la vinification. La majorité de ce composé est extraite entre le 2ème et le 5ème jour de fermentation, lorsque l'activité levurienne et la production d'éthanol sont maximales (Siebert et Solomon 2011). Une étude a montré que seulement 10% de la rotundone présente dans les raisins étaient extraits lors de la fermentation, et que 6% se retrouvaient dans le vin en bouteille (Caputi, et al. 2011). L'addition d'éthanol ou de sucre équivalent à un **enrichissement** de 4%. vol. permet d'atteindre un pourcentage d'extraction supérieur à 19% en fin de fermentation alcoolique (Zhang, et al. 2017).

Des pertes importantes sont observées au cours du **soutirage**, de la **filtration** et du **collage** des vins en lien vraisemblablement avec la nature hydrophobe de la molécule (**LogP** prédit = 4.98) et ses propriétés à se lier à d'autres particules (Caputi, et al. 2011). Le LogP, aussi appelé Log Kow, est une mesure de la solubilité différentielle d'une molécule dans l'octanol et dans l'eau. Cette valeur est égale au logarithme du rapport des concentrations de la substance étudiée dans l'octanol et dans l'eau. Une valeur positive et élevée signifie que la molécule est plus soluble dans l'octanol et dans l'eau et donc possède un caractère hydrophobe ou lipophile.

Compte tenu de sa localisation dans la pellicule, les techniques de vinification sont susceptibles d'avoir une incidence sur la concentration finale dans le vin. Une étude a permis de comparer l'impact de plusieurs **techniques de vinification** et de **variables fermentaires** sur la rotundone (Geffroy, et al. 2017). Aucune des techniques expérimentées, y compris l'addition d'enzymes de macération et une macération préfermentaire à froid de 72 heures à 4 °C, n'a permis de favoriser la rotundone dans les vins par rapport à un témoin vinifié à 25 °C, pendant 8 jours. Au contraire, une diminution de 20% a été observée pour les modalités macération semi-carbonique, fermentation à l'aide d'une souche de *Saccharomyces uvarum*, fermentation à 30 °C pendant 8 jours, et fermentation à 25 °C pendant 8 jours suivie d'une macération post-fermentaire de 6 jours. Il est communément admis qu'une augmentation de la durée et de la température de macération favorise l'extraction des composés pelliculaires. Dans les conditions expérimentales de cette étude, il semblerait que la rotundone ait été absorbée par les lies dont la précipitation a été davantage marquée pour ces traitements. Les faibles concentrations en rotundone mesurées pour les modalités thermovinifiées et vinifiées en rosé s'expliquent par le retrait précoce des pellicules.

La molécule est très **stable**, évolue peu dans les vins en bouteille même après plusieurs années de stockage et **n'est pas « scalpée »** par l'obturateur (Herderich, et al. 2012).

Chapitre 2 :

Aspects sensoriels liés à la rotundone et appréciation par les consommateurs

Hormis la mise en évidence d'une anosmie spécifique à la rotundone lors des travaux initiaux ayant abouti à sa découverte, aucun élément sensoriel n'était disponible jusqu'en 2014 sur cette molécule. Nous avons ainsi cherché à vérifier s'il existait une relation entre l'intensité des notes poivrées à la dégustation et la concentration en rotundone des vins. Afin de limiter les effets liés à la matrice et au vieillissement, l'étude a été réalisée sur 21 vins de Gamay N du millésime 2013. Le choix de ce cépage exprimant souvent un caractère poivré et représenté sur l'ensemble du territoire français, nous a permis d'obtenir une importante diversité de concentrations en rotundone. Nous avons ainsi pu mettre en évidence **une corrélation significative positive entre les données analytiques et sensorielles** (R2 = 0.66) (Article 1).

Contrairement à d'autres molécules libres d'origine variétale comme les alkyl-méthoxypyrazines perçues plutôt négativement, les notes poivrées pourraient être recherchées par les consommateurs. Afin d'évaluer l'appréciation de ces notes et d'identifier un profil de consommation pour les vins poivrés, **une étude consommateur a été menée auprès de 87 panélistes**. Sur la base de leur appartenance à des groupes homogènes de profil sensoriel (amylique, lacté, végétal et poivré) déterminés par analyse en composante principales suivie d'une classification ascendante hiérarchique, 4 vins de Gamay sur les 21 de l'échantillon ont été sélectionnés pour participer à un **test de classement**.

Il est apparu que **les arômes poivrés n'étaient pas appréciés par l'ensemble des panélistes**, notamment ceux préférant les vins au profil amylique (Article 1). Ces observations suggèrent que la rotundone pourrait être indésirable à partir d'une certaine concentration. Afin d'évaluer cette valeur seuil, une nouvelle étude a été mise en œuvre sur un vin rouge très faiblement pourvu en rotundone, en utilisant la méthodologie du **seuil de rejet par le consommateur ou *consumer rejection threshold* (CRT)** (Prescott, et al. 2005). Les résultats ont montré que le **caractère poivré était perçu positivement** par la plupart des consommateurs à l'exception des jeunes panelistes (Article 2). Parmi les **31% d'anosmique** de notre panel, les panelistes ayant plus de 55 ans sont moins susceptibles de détecter la rotundone. Chez les anosmiques, la rotundone a induit un rejet de l'échantillon complémenté à 25 ng/L ce qui laisse supposer que la rotundone est capable d'induire un stimulus chez cette population.

<u>Article 1:</u>

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A sensory, chemical and consumer study of the peppery typicality of French Gamay wines from cool-climate vineyards

Olivier GEFFROY, Camille BUISSIÈRE,
Valérie LEMPEREUR et Bertrand CHATELET

Résumé : La typicité aromatique de 21 vins de Gamay N produits dans 4 bassins viticoles français a fait l'objet d'une étude analytique, sensorielle et consommateurs. Cette étude a permis de mettre en évidence que les vins de Gamay N produits sous le climat auvergnat, frais et pluvieux au cours de la maturation des raisins, présentaient des notes poivrées et des concentrations en rotundone supérieures. Par ailleurs, une corrélation significative positive a pu être établie entre ces deux paramètres. Parmi les 21 références de l'étude, 4 vins présentant des profils sensoriels distincts (amylique, lacté, végétal et poivré) ont été sélectionnés pour participer à deux études consommateurs menées à Clermont-Ferrand (n=47) et à Paris (n=40). L'analyse des données des tests de classement n'a pas permis de mettre en évidence de différences significatives entre les vins en termes de préférence. Cependant, nos résultats ont permis d'identifier le profil de l'amateur de vins poivrés qui s'apparente à un amateur éclairé possédant un budget vin supérieur au reste du panel.

A SENSORY, CHEMICAL AND CONSUMER STUDY OF THE PEPPERY TYPICALITY OF FRENCH GAMAY WINES FROM COOL-CLIMATE VINEYARDS

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Abstract

Aim: Within the protected designation of origin (PDO) Côtes d'Auvergne, Gamay N wines express unique peppery notes that may reflect high levels of rotundone. We investigated the typicality of these wines by determining their sensory, chemical and consumer profiles.

Methods and results: Twenty-one Gamay N wines from the 2013 vintage from four French wine-growing areas were assessed by a trained sensory panel (n = 8). Principal component analysis and hierarchical clustering of olfactory data were used to describe differences among regions and to select four wines for a consumer study (n = 87). Gamay N wines from Auvergne had more intense peppery notes and higher rotundone concentrations, two characteristics that showed a significant positive correlation. The large variability in rotundone among the 12 wines from Auvergne was attributed to ethanol content, which was correlated to the rotundone levels in the wines. Those who appreciate wines with a peppery sensory profile were generally managers and professionals who are willing to pay more for a bottle of wine.

Conclusion: There were differences in sensory profile and rotundone concentrations in Gamay N wines from cool-climate vineyards. We also identified the consumption profile of those who appreciate peppery wines.

Significance and impact of the study: Our results provide a scientific foundation for Auvergne grape growers to promote the typicality of their wines. This research also identifies the key elements for developing the Côtes d'Auvergne wine range and adapting products to consumer profiles.

Key words: typicality, Gamay N, rotundone, peppery wine, cool climate

Résumé

Objectif: Au sein de l'Appellation d'Origine Protégée (AOP) Côtes d'Auvergne, les vins de Gamay N expriment des caractéristiques poivrées uniques qui pourraient être imputables à la présence de hauts niveaux de rotundone. Afin d'étudier cette typicité, un travail de recherche incluant une étude sensorielle, analytique et consommateur a été mené.

Méthodes et résultats: Vingt-et-un vins de Gamay N du millésime 2013 en provenance de quatre régions viticoles françaises ont été évalués par un jury expert (n = 8). L'analyse en composantes principales et la classification hiérarchique ascendante des données de l'examen olfactif ont été utilisées afin de décrire les différences entre les régions et de sélectionner quatre vins pour participer à deux études consommateur (n = 87). Les vins de Gamay N d'Auvergne présentent des notes poivrées et des concentrations en rotundone supérieures, et une corrélation significative positive a pu être établie entre ces deux paramètres. Afin d'expliquer l'importante variabilité de concentration en rotundone au sein des 12 échantillons auvergnats, la teneur en éthanol des vins a été identifiée comme une variable clé, significativement corrélée au niveau de rotundone dans les vins. Les consommateurs appréciant les vins poivrés s'apparentent à des cadres et possèdent un budget élevé.

Conclusion : Nos résultats ont permis de mettre en évidence des différences de profil sensoriel et de concentrations en rotundone dans les vins de Gamay N en provenance de vignobles au climat frais. Les résultats acquis ont également permis d'identifier le profil de l'amateur de vins poivrés.

Signification et impact de l'étude : Nos résultats sont susceptibles d'aider les producteurs auvergnats à promouvoir la typicité de leurs vins. Ce travail de recherche leur fournit également des éléments clés afin de construire leur gamme et de cibler leurs produits en fonction du profil de consommation.

Mot clés: typicité, Gamay N, rotundone, vin poivré, climat froid

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INTRODUCTION

With almost 29 000 ha of vineyards in 2011 (plantgrape.plantnet-project.org), Gamay N is the eighth most planted grape cultivar in France. It is also named "*Gamay noir à jus blanc*" (black-skinned Gamay with white juice) to distinguish it from tinted Gamay cultivars (i.e. Gamay Fréaux, Gamay de Bouze and Gamay de Chaudenay), which produce grapes with colored pulp as a result of a mutation of Gamay N. Although Gamay N is thought to have first appeared in the village of Gamay near Saint-Sernin-du-Bois in Saône-et-Loire in the 1360s (Johnson, 1989), this hypothesis is unlikely because this cultivar has never been planted in the area (Robinson *et al.*, 2013). Others have suggested that Gamay N or its direct ancestors were introduced from Dalmatia by the troops of Probus, a Roman emperor (Viala and Vermorel, 1902), also improbable because genetic studies have shown that it descends from a cross between Pinot Noir N and Gouais B (Bowers *et al.*, 1999). Early to ripen and productive, Gamay N is scattered across French wine-growing regions and, according to FranceAgriMer (www.franceagrimer.fr), in 2010 represented 20500 ha in the Burgundy and Rhône-Alpes regions (with most of the planted surface area in the Beaujolais area), 2000 ha in the Loire Valley and 1050 ha in South West France. Gamay N can also be found in several small vineyards, in cool-climate areas such as Côtes Roannaises, Côtes du Forez or Côtes d'Auvergne. Gamay N is also the second largest variety planted in Switzerland, after Pinot Noir, with about 1500 ha (Robinson *et al.*, 2013).

In most of the vineyards where it is planted, Gamay N is used to produce soft, colored, pleasant wines with fruity characteristics. These wines are made from grapes that undergo carbonic or semi-carbonic maceration (whole berries) and pre-fermentation heat treatment, except for the 10 Beaujolais crus that aim for more concentrated products and some small vineyards that cannot afford to invest in expensive heating equipment. Carbonic maceration generates higher concentrations of ethyl decanoate, eugenol, methyl and ethyl vanillates, ethyl and vinylguaiacols, ethyl and vinylphenols, ethyl cinnamate and ethyl decanoate (Versini and Tomasi, 1983; Ducruet, 1984; Fondville-Bagnol, 1996), and pre-fermentation heat treatment of grapes produces wines with higher level of ethyl esters, acetates and fatty acids (Geffroy *et al.*, 2015b). Chatelet *et al.* (2014) showed that 3-mercaptohexanol and its acetate may be involved in the blackcurrant aroma of wines made from Gamay N. However, few studies have investigated the varietal aroma of Gamay N wines.

Within the protected designation of origin (PDO) Côtes d'Auvergne, a 400-ha cool-climate vineyard located in Central France near the city of Clermont-Ferrand, Gamay N wines are known to express some unique spicy and even peppery notes. Rotundone, a sesquiterpene discovered in 2008 in an Australian Shiraz wine (Wood *et al.*, 2008), may be responsible for these distinctive flavors. This compound has been identified in an increasing number of vine cultivars, including Grüner Veltliner, Vespolina and Schioppettino in Italy (Caputi *et al.*, 2011; Mattivi *et al.*, 2011), Durif and Graciano in Australia (Herderich *et al.*, 2012) and Duras in South West France (Geffroy *et al.*, 2014).

Geographical influences on wine sensory profiles for a given cultivar have been studied for Sauvignon Blanc (Lund *et al.*, 2009), Malbec (Goldner and Zamora, 2007), Albariño (Vilanova and Vilarino, 2006), Touriga Nacional (Falqué *et al.*, 2004), Riesling (Douglas *et al.*, 2001), Chardonnay (Cliff and Dever, 1996; Schlosser et *al.*, 2005) and Pinot Noir (Cliff and Dever, 1996), but never for Gamay N. Most sensory studies highlight large differences in the sensory profiles of wines among regions of production.

To investigate the peppery typicality of Gamay N wines from the PDO Côtes d'Auvergne, a study was conducted on 21 Gamay N wines from the 2013 vintage from four French viticultural areas (Beaujolais, Loire Valley, South West and Auvergne). This research began with an assessment of the sensory differences among the wines. Standard chemical and rotundone analyses were performed in order to evaluate the variability of rotundone concentrations among the sampled wines and to correlate the peppery scores with the chemical associated with this flavor. Four wines showing distinct sensory profiles (including a peppery one) were chosen to participate in a study to assess consumer preferences. Our research work was inspired by a study conducted by Lund *et al.* (2009) on Sauvignon blanc.

MATERIALS AND METHODS

1. Wines

Vintage has a strong impact on the concentrations of grape-derived aroma compounds and those produced by yeasts, such as ethyl esters, acetates and acids, in wines made using traditional maceration techniques and/or employing pre-fermentation heat treatment of grapes (Geffroy *et al.*, 2015b). For this reason, all 21 wines in the study were chosen from the same vintage (2013). In most French wine-growing areas, the 2013 growing season was characterized by rainy conditions during the vegetative growth of the vines and low water deficits, conditions that should be favorable to high rotundone concentrations in wines (Geffroy *et al.*, 2014). Wines (Table 1) were selected from Auvergne (Côtes d'Auvergne, including the other four red geographical indications of the PDO, i.e. Châteaugay, Madargue, Boudes and Chanturgue), South West (Gaillac), Loire Valley (Touraine, Côteaux d'Ancenis) and Beaujolais (Beaujolais Nouveau, Beaujolais, Beaujolais Villages and Brouilly). Wines were coded by a letter that refers to its region of origin (A for Auvergne, B for Beaujolais, L for Loire Valley and S for South West) and a number (from 1 to 12 for Auvergne, 1 to 6 for Beaujolais, 1 to 2 for Loire Valley and 1 for South West).

Wines were selected on the basis of being predominantly from Gamay grapes (>85%) and not being aged in oak barrels. However, one wine (A-1) was aged in oak barrels and two wines were vinified with fresh oak chips (L-1 and L-2). The retail price of the wines (which was not a criterion for selection), varied from EUR 4.50 to 9.00 VAT inclusive at the winery. With the help of contact people based in each of the four wine-growing regions, all wines were chosen for their typicality and representativeness in terms of quantity produced. The contact people also provided important information on the wines such as the winemaking techniques used, average phenology (i.e. mid-veraison date), and average climatic data for 2013 (from one weather station for Gaillac, two for the Loire Valley, three for Auvergne and seven for Beaujolais). With these climatic data, several indices endorsed by Tonietto and Carbonneau (2002) were calculated for the four regions: the Huglin index or heliothermal index from 1 April to 30 September, the cool night index (FNv-r), the mean air temperature (Tv-r), the maximal air temperature (Txv-r), and the thermal amplitude (Av-r) indices during the veraison-harvest period. Average mid-veraison dates were 25, 31, 25 and 17 August for Auvergne, Beaujolais, Loire Valley and South West, respectively. Indices were calculated on the assumption that harvest took place 45 days after mid-veraison. Cumulative rainfalls during the whole calendar year and during the budburst-veraison and veraison-harvest periods were also calculated. Wineries in Auvergne are very small and one "cuvee" is generally produced from grapes harvested on one single plot, making it possible to compile viticultural and enological data (GPS coordinates, vine spacing, altitude, picking date, use of chaptalization, temperature and length of maceration).

2. Sensory analysis

The initial expert panel was composed of 10 panelists (7 males and 3 females) who had prior experience in wine assessment. Ten training sessions were organized within 2 months prior to descriptive analysis and the panel became familiar with intensity rating of aromatic intensity ('intensity'), wine aroma sensory attributes and defects based on standard references from the Nez du Vin (Jean Lenoir, Carnoux-en-Provence, France) and the AWRI (Adelaide, Australia) as presented in Table 2 along with their corresponding odor reference standards. For in-mouth perceptions, solutions containing different concentrations of glycerol for fatness (0-20 g/L), glucose for sweetness (0-20 g/L), tartaric acid for acidity (0-1.5 g/L), quinine for bitterness (0-15 mg/L), ethanol for alcohol perception (10-15 % vol.) and alum sulfate for astringency (0-2 g/L) were presented to the panel to aid with recognition and discrimination between the different palate sensations. Tannin quality was assessed according to an unpublished, in-house-validated methodology, using three enological tannin preparations (0-1.5 g/L) provided by Oenofrance (Bordeaux, France). This methodology consists in dividing tannins into three different categories with one commercial preparation corresponding to one textural sensation on the palate: hard ("firm" tannins with good quality), green ("sticky" tannins often found in wines made from unripe grapes and that need oxygen to soften), and dry ("grainy" tannins usually extracted from oak and that cannot evolve during aging). After the training

Table 1. Number of wines included in the descriptive sensory, standard chemical and rotundone analyses (n = 21).

Region/PDO	Quantity
Auvergne	**12**
Côtes d'Auvergne	5
Côtes d'Auvergne Châteaugay	3
Côtes d'Auvergne Madargue	1
Côtes d'Auvergne Boudes	2
Côtes d'Auvergne Chanturgue	1
Beaujolais	**6**
Beaujolais Nouveau	1
Beaujolais	3
Beaujolais Villages	1
Brouilly	1
Loire Valley	**2**
Touraine	1
Côteaux d'Ancenis	1
South West	**1**
Gaillac Primeur	1

Table 2 - Aroma attributes selected for descriptive analysis and composition of the corresponding reference standards.

Attribute	Reference standard (quantities[a] or concentration)
Oxidation level	1 drop each of sample 6 (sulfur) and 9 (cauliflower) of Le Nez du Vin *"Les défauts"* for reduced aroma (0 on the rating scale)
	1 drop of sample 2 (overripe apple) of Le Nez du Vin *"Les défauts"* for oxidized aroma (5 on the rating scale)
Defect	1 drop of sample 3 (vinegar) of Le Nez du Vin *"Les défauts"* for acetic acid bacteria spoilage
	1 drop of sample 12 (cork) of Le Nez du Vin *"Les défauts"* for cork taint
	1 drop of sample 10 (horse) of Le Nez du Vin *"Les défauts"* for *Brettanomyces* contamination
Fermentative/amylic	1 drop of sample 5 (banana) of Le Nez du Vin
Fermentative/lactic	10 mL of liquid cream, Délisse
Floral	1 drop of sample 29 (violet) of Le Nez du Vin
Thiol	1 drop each of sample 2 (grapefruit) and 37 (blackcurrant bud) of Le Nez du Vin
Terpenic	1 drop each of sample 12 (strawberry) and 13 (raspberry) of Le Nez du Vin
Spicy/peppery	200 ng/L of rotundone in neutral red wine
Spicy/licorice	1 drop of sample 36 (licorice) of Le Nez du Vin
Green	1 drop of sample 30 (green bell pepper) of Le Nez du Vin
Oaky	1 drop each of sample 48 (toast) and 49 (roasted almonds) of Le Nez du Vin

[a]Quantities specified are those added to 40 mL of neutral red wine.

sessions, two panelists (one male and one female) were excluded from the final panel because they could not detect rotundone and peppery flavors. Specific anosmia to this compound has been reported: for example, approximately 20 % of panelists cannot detect this compound in sensory trials at very high concentrations (4000 ng/L), even in water (Wood *et al.*, 2008). The wine sensory descriptive analysis was performed in accordance with the ISO 11035 (1994) standard in a professional room. A constant volume of 10 mL of each wine was poured in black wine-tasting glasses at 15 °C. Each panelist evaluated each of the 21 wines on 30 June 2014 once in randomized order to rate the intensity of sensory attributes. The intensity of each attribute was scored on a five-point rating scale, on which "0" indicated that the attribute was not perceived and "5" that it was perceived at very high intensity (except for oxidation level whose scale ranged from zero - having a reduced aroma - to five - having an oxidized aroma). For defect, the panelists did not have to describe the nature of the defect but only to rate its intensity if attributes associated with acetic acid bacteria spoilage, *Brettanomyces* contamination or cork taint were perceived. Presentation order of the 21 samples, coded with three-digit codes and served blind, was randomized for each panelist. Both presentation order and number codes were determined using Tastel software (ABT Informatique, France).

3. Standard chemical and rotundone analyses

Conventional enological parameters and rotundone levels were determined for the 21 bottled wines in mid-July 2014, less than one month after the descriptive analysis and consumer study. Alcohol content was measured using an Alcoquick L200 infrared analyzer (Unisensor, Germany) and pH with a Titromatic pH meter (Hachlange, Germany). Total acidity was measured according to the OIV method (OIV, 2009). A Konelab Arena 20 sequential analyzer (Thermo Electron Corporation, USA) was used with enzyme kits to determine volatile acidity (Megazyme, Ireland) and glucose/fructose (Thermo Fisher Scientific, USA). Anthocyanins and total phenolic index (TPI) were quantified according to the techniques described in Ribéreau-Gayon and Stonestreet (1965) and Ribéreau-Gayon (1970), respectively, using an Evolution 100 spectrophotometer (Thermo Electron Corporation, USA). All determinations were carried out in duplicate. Rotundone concentrations in wine were determined by the AWRI, as part of a contract service, using solid phase microextraction-multidimensional gas chromatography-mass spectrometry (Geffroy *et al.*, 2014).

4. Consumer study

Panelists, all of whom were self-declared red wine consumers, were recruited from wine shops, by word of mouth, and, for the Parisian session, via social networks and announcements in leading French wine magazines (*La Revue des Vins de France, Le Figaro Vin*). Remuneration for participating in the study consisted of a bottle of wine and a book. Consumers knew only they were tasting some red wines. They provided demographic information and responded to purchase behavior questions. Socio-economic classification was determined according to French National Institute for Statistics and Economics

(INSEE) categories : farmers, mid-level occupations, managers and professionals, service, sales and support workers, craft and trades workers, elementary occupations, students, and unemployed workers. Two distinct sessions were organized on 1 July and 2 July 2014 in Auvergne, in the city of Clermont-Ferrand (n = 47), and in Paris (n = 40), respectively. The 87 consumers evaluated all four wines, chosen from the 21 included in the sensory study for their distinct sensory profile. The consumer evaluation was not organized in a professional tasting room with booths but in a neutral room with white walls. Consumers were sufficiently apart from each other to ensure that no communication occurred. Samples were anonymous and presentation order was randomized. A constant volume of 10 mL of each wine was poured in transparent wine-tasting glasses at 15 °C. Ranking tests were performed and the consumer panelists first had to carry out an olfactory assessment based on aroma preferences, and then a taste assessment based on flavor and texture preferences (i.e. acidity, balance, bitterness, quantity and quality of tannins). Wines were ranked by the panelists from 1 ("the favorite") to 4 ("the least appreciated"). The panelists were allowed to retaste samples if requested.

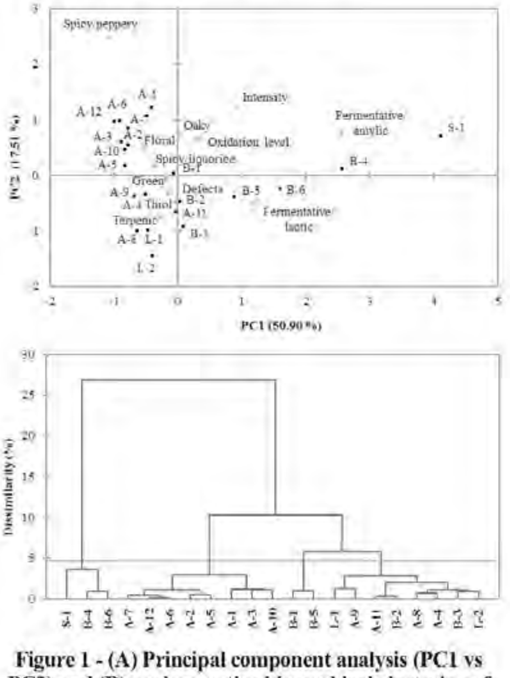

Figure 1 - (A) Principal component analysis (PC1 vs PC2) and (B) agglomerative hierarchical clustering of olfactory data of 21 Gamay N wines from four French wine-growing areas. A: Auvergne (n = 12); B: Beaujolais (n = 6); L: Loire Valley (n = 2); S: South West (n = 1).

5. Statistical treatment

Statistical analyses including linear regressions were conducted using Xlstat software (Addinsoft, France). For each of the 21 wines, olfactory data from the descriptive analysis were first averaged over the panelists, treated with principal component analysis (PCA). In order to select the four wines used in the consumer study, an agglomerative hierarchical clustering (AHC) was performed directly on the 12-olfactory descriptor matrix. Then, a one-way analysis of variance (ANOVA) in which region was the factor and panelists and wines were considered as repetitions was carried out on the data from the sensory analysis. Chemical data were also treated through ANOVA considering the region as a factor and wines as repetitions. For the four wines selected for the consumer study, olfactory and taste data were tested in a one-way analysis of variance (ANOVA). Fisher's least significant difference test was used as a post-hoc comparison of means at $P \leq 0.05$. Consumer data were analyzed using Friedman's test followed by a Nemenyi post-hoc test at $P \leq 0.05$.

RESULTS AND DISCUSSION

1. Sensory analysis

The PCA plot (Figure 1A) shows that four attributes explain the main differences observed between the 21 Gamay N wines of the study : intensity, fermentative/lactic, fermentative/amylic and spicy/peppery. The scores discriminated a group of wines from Auvergne with similar sensory characteristics (A-1, A-2, A-3, A-5, A-6, A-7, A-10 and A-12) and heavier peppery notes. Results of the AHC (Figure 1B) led to the same conclusions. S-1, B-4 and B-6 wines had pronounced fermentative/amylic notes, and B-1 and B-5 showed a fermentative/lactic aroma sensory profile. These fermentative aromatic features are likely related to the specific winemaking techniques used. Several studies, in particular that of Geffroy *et al.* (2015b), have shown that pre-fermentative heat treatment of grapes induces a significant increase in acetates, fatty acids and most ethyl esters when wines are fermented in the liquid phase. The other wines of the study showed less distinctive aroma profiles. Grouped by region of production, wines from Auvergne had more intense peppery characteristics (Table 3) with large variability for this attribute. Wines from the Loire Valley were less expressive and had lower intensity. Produced in the northern-most vineyards of this study, these wines were perceived by the panelists as thinner, more acidic, more reductive and with less sweetness than the other wines of the study. Wines from the Beaujolais and the South West of France showed a quite similar

Table 3 - Sensory attributes of the Gamay N wines rated on a five-point scale and grouped by region of production. Data were averaged over the panelists and over the wines from one region. Standard deviations (SD) refer both to within-panelist and within-wine variabilities with the exception of the South West for which it only refers to within-panelist variability.

Sensory attribute	*P*-value	Auvergne (n = 12)		Beaujolais (n = 6)		Loire Valley (n = 2)		South West (n = 1)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Olfactory									
Intensity	< 0.0001	3.30 b[a]	0.91	3.40 b	0.86	2.60 c	0.84	4.80 a	0.44
Oxidation level	0.045	2.63 a	0.88	2.77 a	0.57	2.00 b	0.94	3.00 a	0.00
Defect	0.349	0.22 a	0.65	0.03 a	0.18	0.30 a	0.95	0.00 a	0.00
Fermentative/amylic	< 0.0001	0.08 c	0.42	0.93 b	1.44	0.00 c	0.00	3.80 a	0.81
Fermentative/lactic	< 0.0001	0.05 b	0.39	1.13 a	1.46	0.40 b	1.27	1.80 a	1.12
Floral	0.060	0.75 a	1.16	0.30 a	0.88	0.10 a	0.32	0.00 a	0.00
Thiol	0.360	0.15 a	0.52	0.37 a	0.81	0.30 a	0.67	0.00 a	0.00
Terpenic	0.640	0.85 a	1.05	1.07 a	0.94	1.20 a	1.03	0.80 a	0.63
Spicy/peppery	< 0.0001	1.53 a	1.51	0.53 b	1.12	0.10 b	0.32	0.00 b	0.00
Spicy/licorice	0.383	0.65 a	1.02	0.33 a	0.66	0.60 a	1.07	0.20 a	0.45
Green	0.207	0.20 a	0.68	0.00 a	0.00	0.40 a	0.84	0.00 a	0.00
Oaky	0.715	0.48 a	1.04	0.33 a	0.66	0.20 a	0.42	0.00 a	0.89
Taste									
Fatness	< 0.01	1.98 a	0.91	2.17 a	0.87	1.10 b	0.99	2.60 a	1.14
Sweetness	0.024	0.48 ab	0.79	1.03 a	1.35	0.30 b	0.67	1.40 a	1.01
Acidity	0.026	1.20 b	1.22	0.80 b	1.06	2.10 a	1.19	0.60 b	1.14
Astringency	0.478	2.10 a	0.97	2.37 a	0.89	1.90 a	0.88	2.20 a	0.73
Bitterness	0.707	0.12 a	0.52	0.03 a	0.18	0.20 a	0.63	0.20 a	0.34
Alcohol perception	0.609	1.20 a	1.29	1.03 a	1.19	0.80 a	1.13	0.60 a	1.10
Hard tannins	0.109	1.55 a	1.11	1.87 a	0.97	0.90 a	1.10	0.60 a	1.01
Green tannins	0.846	0.53 a	0.83	0.70 a	1.02	0.70 a	0.67	0.60 a	0.74
Dry tannins	0.150	0.23 a	0.65	0.00 a	0,00	0.40 a	0.66	0.16 a	0.37

[a]Means with the same letter within a row are not significantly different according to the least significant difference test at $P \leq 0.05$.

sensory profile with more intense fermentative/lactic and fermentative/amylic notes. For this last attribute, the wine from the South West, which was the most intense and rather aromatically simple, received the highest score.

2. Standard chemical and rotundone analyses

Together with the wine from the South West, wines from Beaujolais were characterized by higher pH associated with lower acidity (Table 4). In accordance with the sensory observations, wines from the Loire Valley showed the lowest pH. Among the wines from Auvergne, large variability was observed for most studied chemical attributes (i.e. alcohol content, total acidity). The 2013 vintage was late in ripening due to rainy weather conditions in early October in Auvergne. Therefore, the observed variability might reflect differences in behavior among winegrowers : those who chose to harvest grapes early to limit *Botrytis cinerea* severity and those who took the risk to wait longer until better ripening. For rotundone (Figure 2), there were no differences between the wines from Beaujolais, Loire Valley and South West. The Gamay N wines from Auvergne had higher concentrations in this aroma compound, corroborating the results of the descriptive analysis. Among the 12 wines from this region, four had a rotundone content greater than 100 ng/L, with a maximum of 142 ng/L measured in wine A-7. The 12 Gamay N wines from Auvergne were produced using traditional winemaking techniques with maceration durations ranging from 5 to 31 days, whereas the other wines in the study all contained a percentage of wine produced by semi-carbonic (whole berries) maceration or pre-fermentation heat treatment of grapes. As rotundone is a hydrophobic compound that is extracted from the berries during the first days of alcoholic fermentation by the solvent effect of ethanol (Siebert and Solomon, 2011), semi-carbonic maceration and pre-fermentation heat treatment do not *a priori* promote

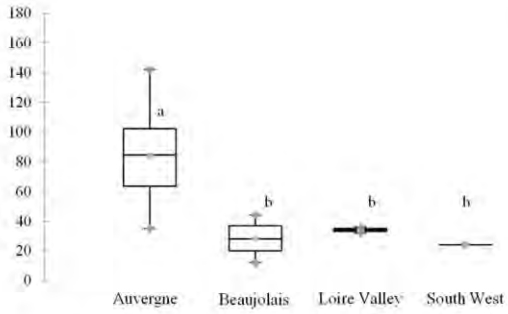

Figure 2 - Rotundone concentrations in Gamay N wines according to region of production.
Different letters indicate means significantly different at $P \leq 0.05$ by Fisher test.

the production of wines with high rotundone concentrations. Therefore, it remains to be determined if the higher levels found in the wines from Auvergne reflect a real "terroir" effect or arise from specific winemaking techniques. Several hypotheses in regard to climate conditions have been proposed to explain the variation in rotundone levels among vintages, vineyards or within the same plot. According to Caputi *et al.* (2011), the cool and rainy vintages or vineyards are particularly favorable to the accumulation of rotundone in grapes. According to Geffroy *et al.* (2014), water status experienced by the vine late in the growing season is a key variable in inter-vintage variation in rotundone. More recently, Zhang *et al.* (2015) showed that berry temperature exceeding 25 °C during the veraison-to-harvest period negatively affects the rotundone concentration in Shiraz and that temperature was one of the main determinants of rotundone in grape berries. Climatic indices presented in Table 5 help compare the climate in the four viticultural areas accurately. Auvergne is 1) the coolest vineyard over the whole wine-growing season and the ripening period, and 2) the wettest during the veraison-harvest period, although it is the driest over the whole calendar year. Its "terroir" via its climate component is therefore the most likely factor explaining the differences observed between the wines from Auvergne and the other regions of the study. These differences may be further amplified by the winemaking techniques.

3. Relationship between rotundone and spicy/peppery aroma

Wine is a complex matrix containing hundreds of volatiles that can act in synergy, and in which ethanol and aroma compounds can operate as a buffer or as a mask (Ferreira, 2012). Despite the complexity of wine aroma and considering the expected high concentrations in esters and acetates in wines made using alternative winemaking techniques, a significant correlation (Figure 3) was observed between peppery aroma scores and rotundone concentration in wine ($r^2 = 0.66$). This conclusion corroborates observations made by Ferreira (2012), who described rotundone as one of the 16 most "impacting" aroma compounds in wine. The observed coefficient of determination is comparable to values obtained in previous studies investigating the link between chemical compounds and sensory data. Coefficients of determination ranging from 0.50 to 0.80 have been reported, for example, in white wines made from Albariño (Vilanova *et al.*, 2010), Sauvignon blanc (Lund *et al.*, 2009) and in red wines from Cabernet-Sauvignon and Merlot (Roujou de Boubée, 2000).

4. Variability in rotundone among the samples from Auvergne

There was high variability in rotundone content among the 12 wines from Auvergne, with the lowest value of 35 ng/L measured in wine A-8, and the highest value reaching 142 ng/L in wine A-7. As the 12 wines from Auvergne were each made from a

Table 4 - Conventional enological parameters of the Gamay N wines according to region of production.

Parameter	*P*-value	Auvergne (n = 12)		Beaujolais (n = 6)		Loire Valley (n= 2)		South West (n = 1)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alcohol content (% vol.)	0.322	12.35 a[a]	0.69	12.30 a	0.18	11.68 a	0.46	11.58 a	-
Total acidity (g/L H_2SO_4)	< 0.001	3.96 a	0.27	3.38 b	0.22	4.00 a	0.04	2.82 b	-
pH	< 0.0001	3.48 c	0.08	3.59 b	0.11	3.21 d	0.02	3.85 a	-
Volatile acidity (g/L acetic acid)	0.031	0.43 a	0.13	0.28 b	0.06	0.25 b	0.01	0.21 b	-
Glucose/Fructose (g/L)	0.691	0.64 a	0.91	0.35 a	0.52	0.20 a	0.30	0.00 a	-
Total phenolic index	0.317	51 a	9	55 a	10	41 a	1	52 a	-
Anthocyanins (mg/L)	0.268	234 a	81	307 a	61	232 a	1	264 a	-

[a]Means with the same letter within a row are not significantly different according to the least significant difference test at $P \leq 0.05$.

Table 5 - Characterization of the 2013 vintage in the four regions of the study according to several climatic indices and cumulative rainfall calculated over the whole calendar year and the budburst-veraison and veraison-harvest periods.

Region	Huglin index (IH)	Cool night index (FNv-r)	Mean air temperature (Tv-r)	Maximal air temperature (Txv-r)	Thermal amplitude (Av-r)	Cumulative rainfalls (mm)		
						01/01 - 31/12	Budburst - veraison	Veraison - harvest
Auvergne	1490	11.4	15.2	20.8	9.4	599	366	105
Beaujolais	1731	11.8	15.5	21.0	9.2	801	419	88
Loire Valley	1574	12.4	16.8	22.1	9.7	722	232	57
South West	1909	12.3	17.8	24.7	12.4	782	347	48

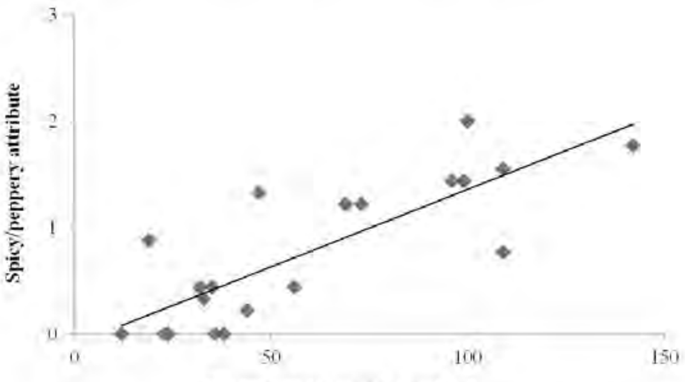

Figure 3 - Relationship between rotundone concentration in wines and their spicy/peppery score rated on a five-point scale (n = 21). Linear regression model: y = 0.0146x – 0.0969; $P < 0.0001$; $r^2 = 0.66$.

single vineyard plot, we mapped wine rotundone concentrations according to the plots from which the grapes were primarily sourced (Figure 5). The rotundone distribution does not appear to be spatially structured. Although soil characteristics and climatic data likely play a role, "terroir" may not be the dominant factor explaining the differences observed. Based on previous studies on rotundone, the observed variability may have a multifactorial explanation involving parameters related to plant material (Siebert and Solomon, 2011; Geffroy *et al.*, 2015a), level of ripening, water deficit and wine-growing techniques such as leaf removal (Geffroy *et al.*, 2014) and winemaking techniques. Several viticultural and enological data were collected for the 12 plots from Auvergne. One wine from Auvergne (A-4) was chaptalized, which led to an increase in ethanol concentration in the final wine (+1 % vol.). The only parameter that accounted for the variability among the wines was alcohol content. After removing from the treatment the data associated with the chaptalized wine, there was a significantly positive correlation at $P < 0.05$ (Figure 4) between this alcohol content and rotundone ($r^2 = 0.44$). On the one hand, this result confirms previous studies by Geffroy *et al.* (2014) and Caputi *et al.* (2011), which have shown that rotundone concentration increases with the level of ripening. On the other hand, higher ethanol concentrations may have induced higher extraction of rotundone from the skin during the alcoholic fermentation. However, the effect of the differences in ethanol should be minimal because studies have shown that the impact of ethanol on the extraction of hydrophobic compounds, such as proanthocyanidins, do not exceed a few percent (Canals *et al.*, 2005).

5. Wines selected for the consumer study and composition of the panel

The sensory characteristics of the four wines selected for the consumer study (S-1, B-1, A-6 and A-8) are shown in Table 6. S-1 whose sensory profile can be qualified as 'amylic', had higher aromatic intensity and heavy fermentative/amylic and fermentative/lactic notes. B-1 had a more complex profile marked by significantly higher 'lactic' notes. A-6 and A-8 were characterized by 'peppery' and 'green' notes,

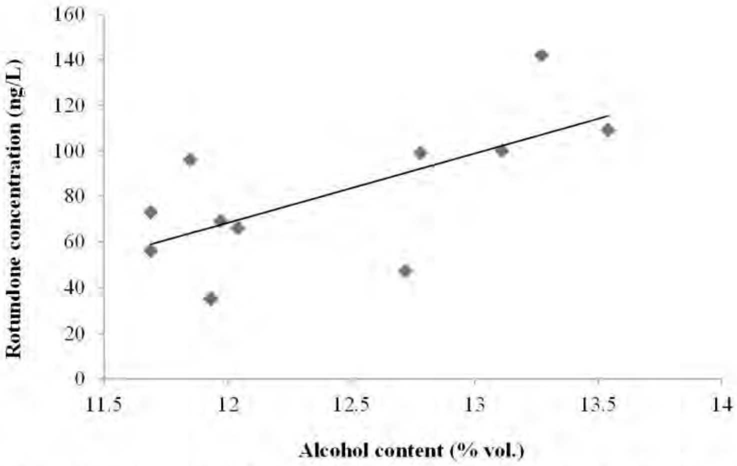

Figure 4 - Relationship between alcohol content and rotundone concentration in wines from Auvergne (n = 11). One data point from a chaptalized wine was removed from the treatment. Linear regression model: y = 30.49x -298; $P < 0.05$; $r^2 = 0.44$.

Table 6 - Sensory attribute scores of the four wines chosen for the consumer study based on a five-point rating scale and rotundone concentration (n = 8).

Attribute	*P*-value	S-1		B-1		A-6		A-8	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Olfactory									
Intensity	0.004	4.43 a[a]	0.82	2.86 b	1.17	3.29 b	0.75	2.57 b	1.05
Oxidation level	0.644	3.00 a	0.00	2.80 a	0.50	2.60 a	0.55	2.00 a	0.96
Defect	0.130	0.00 a	0.00	0.00 a	0.00	0.00 a	0.00	0.43 a	0.00
Fermentative/amylic	< 0.001	3.43 a	1.94	0.14 b	0.00	0.00 b	0.00	0.00 b	0.00
Fermentative/lactic	0.016	1.14 a	1.51	1.43 a	1.21	0.00 b	0.00	0.00 b	0.00
Floral	0.224	0.00 a	0.00	0.57 a	1.03	0.00 a	0.00	0.71 a	0.82
Thiol	0.649	0.00 a	0.00	0.29 a	0.51	0.00 a	0.00	0.14 a	0.41
Terpenic	0.924	0.43 a	0.55	0.80 a	0.98	0.57 a	0.82	0.57 a	0.52
Spicy/peppery	< 0.001	0.00 b	0.00	0.86 b	1.03	2.57 a	1.37	0.43 b	0.52
Spicy/licorice	0.801	0.14 a	0.41	0.00 a	1.03	0.14 a	0.41	0.14 a	0.41
Green	0.039	0.00 b	0.00	0.00 b	0.00	0.00 b	0.00	1.14 a	1.25
Oaky	0.791	0.43 a	0.41	0.14 a	0.00	0.43 a	0.41	0.14 a	0.00
Taste									
Fatness	0.635	2.29 a	1.17	2.29 a	1.05	2.29 a	0.82	1.71 a	1.03
Sweetness	0.210	1.00 a	1.47	0.57 a	0.84	0.14 a	0.41	0.14 a	0.00
Acidity	0.795	1.14 a	1.51	1.14 a	1.03	1.14 a	1.51	1.71 a	1.21
Astringency	0.049	1.71 a	0.71	1.86 a	0.71	2.00 a	0.73	0.71 b	0.75
Bitterness	0.542	0.14 a	0.41	0.00 a	0.00	0.00 a	0.00	0.29 a	0.82
Alcohol perception	0.770	0.43 a	0.00	1.00 a	1.03	0.86 a	1.26	1.00 a	0.98
Hard tannins	0.098	1.23 a	1.17	1.86 a	1.17	1.43 a	0.98	0.43 a	0.84
Green tannins	0.840	0.86 a	1.55	0.71 a	0.82	0.43 a	0.52	0.71 a	0.41
Dry tannins	0.152	0.00 a	0.00	0.00 a	0.00	1.00 a	0.98	0.28 a	0.84
Rotundone (ng/L)	-	24	-	38	-	100	-	35	-

[a]Means with the same letter within a row are not significantly different according to the least significant difference test at $P \leq 0.05$.

Figure 5 - Map of the grape sources for the 12 wines from Auvergne and the rotundone concentrations in those wines. The PDO areas of the four red wine geographical indications (Madargue, Châteaugay, Chanturgue and Boudes) are also shown.

respectively. A-8 had the lowest astringency level among the four wines.

6. Consumer preferences

Demographic information from the Clermont-Ferrand and Paris wine consumers is given in Table 7. Marked differences in terms of panel composition and consumption habits were observed between the two cities. The Parisian panel was younger, included more women, and was mainly composed of managers and professionals. Panelists in Paris had more regular wine consumption habits with 78 % of them drinking wine 3 to 4 times a week, and spend more money on wine in comparison with the consumers from Clermont-Ferrand. Pooled together, the two panels showed a globally well balanced age and gender distribution. Results given in Table 8 show that there were few differences in terms of consumer preferences between the two cities. We therefore pooled the two panels. Regarding the olfactory assessment of the wines, there were no significant differences according to Friedman's test between the four wines. A lack of consensus was observed for wine S-1 as it was rated 37 and 27 times as the favorite and the least appreciated, respectively. The group who judged this wine as the favorite was mainly composed of consumers older than 45 years and contained a larger proportion of females. In the same way, no general agreement was observed for wine A-6, and consumers who rated the 'peppery' wine as the most appreciated were managers and professionals who are willing to pay more for a bottle of wine. It is important to notice that a large percentage of consumers who preferred A-6 was between 35 and 45 years old. The group of consumers who preferred the 'amylic' wine frequently rated the 'peppery' wine as the least appreciated and vice versa. This led us to think that there is an opposition between the consumers who preferred the 'amylic' and the 'peppery' wines. Due to the specific anosmia reported for rotundone, it would have been interesting to investigate whether consumers who preferred or did not appreciate wine A-6 were able to perceive its peppery notes. However, this type of investigation would require a specific tasting session using water solutions spiked with rotundone at several concentrations. Wines A-8 and B-1 were less frequently cited as the favorite and/or least appreciated, being rated 34 times out of 87 as the second and third favorite wines, respectively, thus eliciting a more middle-of-the-road judgment. Those who preferred these two wines were mainly consumers younger than 35 years old. Differences were significant and more marked for taste evaluation, and A-8, which presents the lowest

Table 7 - Demographic information on the Clermont-Ferrand (n = 47) and Paris (n = 40) panelists.

Demographic information		Clermont-Ferrand	Paris	Pooled panels
Gender	Male	77%	55%	67%
	Female	23%	45%	33%
Age (years)	18-24	2%	15%	8%
	25-34	6%	45%	24%
	35-44	19%	20%	20%
	45-54	32%	10%	22%
	>55	41%	10%	26%
Socio-economic classification[a]	Farmers	4%	0%	2%
	Middle-level occupations	18%	3%	10%
	Managers and professionals	49%	72%	62%
	Service, sales and support workers	16%	2%	9%
	Craft and trades workers	11%	15%	13%
	Elementary occupations	2%	0%	1%
	Students	0%	8%	3%
	Unemployed workers	0%	0%	0%
Wine preference	Red	89%	90%	90%
	White	11%	10%	10%
	Rosé	0%	0%	0%
Wine consumption (frequency)	Once a day	24%	5%	15%
	3-4 times a week	39%	78%	57%
	Once a week	28%	17%	23%
	Twice a month	9%	0%	5%
	Once a month	0%	0%	0%
Average price spent on bottle	<€3	0%	0%	0%
	€3 to €5	21%	2%	12%
	€5 to €10	49%	35%	42%
	>€10	30%	63%	46%

astringency level, was the only wine listed as favorite.

CONCLUSION

This study was conducted on 21 Gamay N wines from four French viticultural areas to better comprehend the aromatic typicality of Gamay N wines from cool-climate vineyards (PDO Côtes d'Auvergne). In comparison with the other regions in the study, wines from Auvergne were characterized by more intense peppery notes with higher rotundone concentrations. These sensory features are likely to be due to a "terroir" effect with Auvergne being the coolest vineyard over the whole wine-growing season and the ripening period, and the wettest during the veraison-harvest period. There was a significantly positive correlation ($r^2 = 0.66$) between spicy/peppery notes and analyzed rotundone levels. Within the PDO Côtes d'Auvergne, there was high variability in rotundone concentrations, which was attributed to the alcohol content of the wines, a factor that was significantly positively correlated with rotundone ($r^2 = 0.44$). Consumer studies performed in two cities (Paris and Clermont-Ferrand) on four wines showing distinct sensory profiles ('amylic', 'lactic', 'peppery' and 'green'), provided interesting conclusions. While no significant differences were observed between the wines for the olfactory assessment, A-8 – the wine with the lowest astringency level – was the only wine of the four preferred in the taste assessment. There was an opposition between the consumers who preferred the 'amylic' and the 'peppery' wines. Those who appreciate wines with a peppery sensory profile were generally managers and professionals who are willing to pay more for a bottle of wine. From a commercial and sales development point of view, these results that scientifically investigated the typicality and originality of Gamay N wines from Auvergne should assist winegrowers from this region in promoting their wines. They allow them to take advantage of the knowledge on rotundone obtained in another wine-growing regions with the aim to produce wines with a desired level of peppery aroma. The consumer study provides the key parameters for developing the Côtes d'Auvergne Gamay N wine range and adapting the products to consumer profiles.

Table 8 - Results of the two consumer studies held in Clermont-Ferrand (n = 47) and Paris (n = 40). Wines were ranked by the panelists from 1 « the favorite » to 4 « the least appreciated ».

Panel	Results	Wine / sensory profile			
		S-1 'Amylic'	B-1 'Lactic'	A-6 'Peppery'	A-8 'Green'
Clermont-Ferrand	**Olfactory evaluation**				
	Sum of the ranks	104 a[a]	130 a	129 a	107 a
	Taste evaluation				
	Sum of the ranks	118 ab	125 ab	132 b	95 a
Paris	**Olfactory evaluation**				
	Sum of the ranks	97 a	105 a	105 a	92 a
	Taste evaluation				
	Sum of the ranks	111 a	102 a	101 a	86 a
Both panels (pooled)	**Olfactory evaluation**				
	Sum of the ranks	201 a	235 a	234 a	199 a
	Number of times rated as the favorite	37	8	21	21
	Number of times rated as the least appreciated	27	19	27	14
	% of consumers who are female when favorite	38	12	29	33
	% of consumers who are managers and professionals when favorite	53	62	67	60
	% of consumers who are older than 45 years when favorite	59	13	43	38
	% of consumers who are between 35 and 45 years old when favorite	20	37	33	10
	% of consumers who are younger than 35 years when favorite	21	50	24	52
	% of consumers who pay more than €5 when favorite	84	75	90	76
	% of consumers who pay more than €10 when favorite	38	25	62	43
	Number of times rated as the least appreciated when A-6 (peppery) is rated as the favorite	12	5	-	4
	Number of times rated as the least appreciated when S-1 (amylic) is rated as the favorite	-	9	19	9
	Taste evaluation				
	Sum of the ranks	229 b	227 b	233 b	181 a

[a]Different letters within the same row indicates sums of the ranks significantly different according to the Nemenyi test at $P \leq 0.05$.

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Article 2:

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Can a certain concentration of rotundone be undesirable in Duras red wine? A study to estimate a consumer rejection threshold for the pepper aroma compound

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Résumé : Cette étude visait à établir un seuil de rejet pour la rotundone en utilisant la méthodologie proposée par Prescott, et al. (2005) pour l'étude des molécules responsables des goûts de bouchon. Les anosmiques, détectés à l'aide de tests triangulaires, représentaient 31% des panélistes. Pour cette population, le vin complémenté à 25 ng/L a été significativement rejeté ce qui suggère que la rotundone est susceptible d'induire un stimulus chez les anosmiques. Pour les autres sujets, aucun seuil de rejet n'a pu être mis en évidence. Nos résultats soulignent que la rotundone est perçue, de manière neutre ou positive par les consommateurs dans la plupart des cas, à l'exception des jeunes panélistes qui préfèrent les vins non poivrés.

Abstract

Background and Aims: Rotundone is responsible for peppery aroma in wine. These notes are not appreciated by all consumers which indicates that rotundone might be considered as a taint above a certain concentration.

Methods and Results: Consumers (n = 62) received pairs of samples consisting of a base wine and the base wine spiked with an ascending concentration of rotundone and were asked to indicate which sample they preferred. Anosmic respondents detected through triangle tests represented 31% of the panellists. For these panellists, the wine spiked at the lowest concentration was significantly rejected suggesting that rotundone might induce a trigeminal sensation and/or is involved in molecular mechanisms of flavour reduction. For remaining panellists, we were not able to determine any consumer rejection threshold. We identified three clusters of consumer profiles. The first cluster preferred a moderate concentration of rotundone and rejected a high concentration. The second cluster, mainly composed of young consumers, preferred the Control. The last group appreciated peppery wines especially at high concentration.

Significance of the Study: Our results may assist grapegrowers producing Duras and other cultivars, where rotundone makes a sensory contribution, to adapt their products to consumer profiles. They also open new fields of investigation into mechanisms involved in specific anosmia to rotundone.

Keywords: *consumer preference, consumer rejection threshold (CRT), Duras red wine, peppery flavour, rotundone*

Introduction

Rotundone is a sesquiterpene originally isolated from the tubers of the grassy weed nutgrass (*Cyperus rotundus)* by Kapadia, et al. (1967). More recently, rotundone has been found in various plants and plant products (Wood, et al. 2008), such as white and black peppers (*Piper nigrum*), marjoram (*Origanum majorana*), oregano (*Origanum vulgare*), geranium (*Pelargonium alchemilloides*), rosemary (*Rosmarinus officinalis*), saltbush (*Atriplex cinereal*), basil (*Ocimum basilicum*), thyme (*Thymus vulgaris*) and grape and wine (*Vitis vinifera* L. cv. Shiraz). Up to day, it has been identified in a wide range of grape cultivars including Pinot Noir, Durif, Graciano, Riesling (Herderich, et al. 2012), Duras (Geffroy, et al. 2014), Gamay (Geffroy, et al. 2016), Malbec, Abouriou (Cullere, et al. 2016), Vespolina, Schioppettino and Gruener Veltliner (Caputi et al. 2011). Recent research showed that two key polymorphisms in a newly discovered allele of the *Vitis vinifera* TPS24 gene were responsible for the production of α-guaiene (Drew, et al. 2016). As rotundone is produced though simple aerial or enzymatic oxidation of α-guaiene (Takase, et al. 2016), these polymorphisms are likely to explain the differences in rotundone concentration observed between cultivars. The compound also plays a significant role in the flavour of oak aged spirits with rotundone increasing with aging time (Genthner 2014). During the same study rotundone was also found in un-aged tequila, which suggests that the compound may also be present in the agave plant.

Rotundone was reported in grape leaves and stems (Capone, et al. 2012) and more recently in Shiraz flower caps at pre-veraison (Zhang, et al. 2016). The compound does not appear to be translocated through the phloem tissues and the hypothesis of a in situ production is more likely (Geffroy, et al. 2016,Zhang, et al. 2016). Viticultural and environmental factors have a substantial impact on rotundone concentration in grape berries and in finished wines. Cool and wet vintages promote the production of red wines with a higher rotundone concentration (Caputi, et al. 2011,Zhang, et al. 2015). Within a single vineyard, large spatial variability in rotundone associated with variation in the land underlying the vineyard and vine water status were reported (Geffroy, et al. 2015,Scarlett, et al. 2014,Zhang, et al. 2015). Patterns of this spatial variation are temporally stable from year to year (Bramley, et al. 2017).

Clonal differences in rotundone concentration were identified among certified clones of Duras (Geffroy, et al. 2015), Shiraz (Siebert and Solomon 2010) and Grüner Veltliner (Caputi, et al. 2011). The compound might be involved in the vine's natural defence mechanisms as a positive correlation was identified between rotundone and severity of powdery mildew (PM) on clusters (Geffroy, et al. 2015). Rotundone concentration increases with the ripening of the fruit (Caputi, et al. 2011,Geffroy, et al. 2014,Logan 2015). It is enhanced by irrigation and lowered by leaf removal in the bunch zone (Geffroy et al. 2014). The spraying of 1-naphthaleneacetic acid, a plant growth regulator (Davies, et al. 2015) and the implementation of a viticultural system combining irrigation and on-vine grape drying with cutting of the fruit bearing cane (Geffroy, et al. 2016) were successfully tested to enhance rotundone in vineyard.

Rotundone is mainly located in the grape exocarp (Caputi, et al. 2011,Siebert and Solomon 2010) and most of it is extracted from the berries between days 2 and 5 of fermentation (Siebert and Solomon 2010). Winemaking techniques and fermentation variables have an impact on rotundone in finished wines (Geffroy, et al. 2017). Compared to a traditional maceration wine, wines made from a rosé vinification and from thermovinification treatments, which involved a pre-ferment removal of skins, resulted in a low rotundone concentration. None of the studied treatments including the use of macerating enzymes and the increase in temperature or time of maceration, resulted in enhanced rotundone concentration.

The compound is potent and has an aroma detection threshold of 8 ng/L in water and 16 ng/L in red wine (Wood, et al. 2008). It has been described as one of the 16 most important aroma compounds in wine (Ferreira 2012). Specific anosmia has been reported for rotundone (Wood, et al. 2008) and during the first sensory trials 20 to 25% of panellists could not detect this compound at high concentration in water. A significant correlation (r2 = 0.66) was identified between peppery aroma scores and rotundone concentration in 21 Gamay wines (Geffroy, et al. (2016). During the same study on consumer preference, the cluster of consumers who preferred peppery wines frequently rated a wine with an amylic wine as the least appreciated and vice versa. This cluster was mainly composed of managers who are willing to pay more for a bottle of wine. When assessing Chinese consumers' liking responses for red wines, the cluster of consumers preferring peppery wines represented 20% of the whole population while for 49% of the panellists the black pepper attribute was negatively related to liking (Williamson, et al. 2012). These results suggest that rotundone might be considered as a taint by certain consumers or that rotundone might be undesirable above a certain concentration.

The consumer rejection threshold (CRT) approach has been used to determine the concentration of a compound which gives a significantly lower preference. Based on a novel application of the paired preference test, the method has been applied to answer several off-flavour questions in wine, such as 2,4,6 trichloroanisole (Prescott, et al. 2005), phenolic substances (Yoo, et al. 2011), 1,1,6-trimethyl-dehydronaphthalene (Ross, et al. 2014), ethyl phenyl-acetate and phenyl acetic acid (Campo, et al. 2012) and 1,8-cineole (Saliba, et al. 2009). According to Francis and Williamson (2015), the approach provides more generally a valuable measure as to when consumers will respond negatively to a flavour.

The aim of the current study was to determine a CRT for rotundone in a Duras red wine according to the methodology established by Prescott, et al. (2005). Duras is one the most planted red grape cultivars in the Protected Designation of Origin Gaillac in the south-west of France. As this grape cultivar is known to be a regular rotundone producer with a final concentration in wine, in most cases, over its detection threshold

(Geffroy, et al. 2014,Geffroy, et al. 2016,Geffroy, et al. 2017,Geffroy, et al. 2015), the base wine spiked with several concentration values of rotundone, was made following a viticultural and oenological process conceived to minimise rotundone and therefore having a negligible concentration of this compound in finished wines. A questionnaire was used to assess whether there are any differences in preference for rotundone across demographic and level of knowledge variables. Anosmic respondents were also identified using a triangle test.

Material and methods

Panellists

The panel was composed of 49 wine consumers and 13 wine professionals (i.e. winemakers, winegrowers, estate managers). Wine consumers, all of whom were self-declared regular consumers of red wine (at least once per month), were recruited in the Toulouse area (south-west of France) through an advertisement published in the regional daily newspaper. Wine professionals from the Gaillac Protected Designation of Origin were recruited via the local association of winegrowers and winemakers. Remuneration for participating in the study consisted of a bottle of wine. The only information about the samples provided to the participants was that they were evaluating some red wines. They were asked to provide some demographic information and to respond to several questions regarding their purchase behaviour and their knowledge of peppery notes at the end of the tasting session. The questionnaire also contained ample space for free comments. The panel included 28 women and 34 men, and the age of panellists was between 18 and 24 (23%), 25 and 34 (26%), 35 and 44 (16%), 45 and 54 (19%) and over 55 (16%). Most of the panellists were managers (39%) and employees (29%) according to French National Institute for Statistics and Economics (INSEE) categories, and 89% reported consuming wine at least once per week. Subjects reported consuming wine for a mean of 17.9 years (standard deviation = 13.9) and described their level of knowledge as 'experts' (21%), 'knowledgeable wine drinkers' (31%) and 'interested in wine' (48%). The label 'expert' was only used for wine professional panellists. Average prices spent on a bottle were €3–5 (16%), €5–10 (73%) and >€10 (11%). The questionnaire revealed that 79% of the panellists had previously heard of peppery notes in wine and that 69% of them appreciated this kind of flavours.

Base wine and other materials

To have a negligible level of rotundone, the base wine used for this study was made in our experimental cellar (Lisle sur Tarn, France) following a special process. Duras grapes (100 kg) from the 2015 vintage – a hot and dry vintage not favourable to the expression of intense peppery notes in wines – were sourced from a non-irrigated vineyard experiencing a moderate to severe water deficit. A two-face leaf removal was performed at mid-veraison and harvest took place early in the maturation period exactly 38 days after mid-veraison date. After destemming and crushing, grapes received a sulfur dioxide addition (40 mg/L) using a 10% bisulfite liquid solution (Solution 10, Laffort, Bordeaux, France). The must was inoculated with 200 mg/L rehydrated active dried *Saccharomyces cerevisiae* yeast (Lalvin Rhône 2056, Danstar Ferment, Zug, 91 Switzerland) and 300 mg/L of diammonium phosphate were added. Grapes were fermented on skins for 8 days at 25°C controlled temperature, and to minimise the extraction of skin compounds (i.e. rotundone), a single punch down per day was performed during the 4 first days of fermentation. Then the wine was racked and inoculated with lactic acid bacteria (Lalvin 31, Danstar Ferment). When the concentration in malic acid, determined using an enzymatic kit (Thermo Fisher Scientific, Waltham, MA, USA), was less than 0.2 g/L, the wine was racked and 50 mg/L of sulfur dioxide was added. The wine was stored in the cellar for 3 months at room temperature and then was tartrate stabilised (1 month at 0°C). Prior to bottling, the wine was filtered through a cartridge filter (Pall France, Saint Germain-en-Laye, France) equipped with 5 and 1 μm filtration cartridges (Prédel, Saint-Loubès, France). Classical oenological analysis of the bottled wine revealed: alcohol concentration 12.1% (Alcoquick L200 infralyser, Unisensor, Karlsruhe, Germany), pH 3.66 (Titromatic pH meter, Hachlange, Düsseldorf, Germany), a TA 4.23 g/L of tartaric acid measured according to the Organisation Internationale de la Vigne et du Vin (OIV) method (Organisation Internationale de la Vigne et du Vin 2009), 0.1 g/L of glucose +fructose, and volatile acidity 0.36 g/L acetic acid (enzymatic kits provided by Megazyme, Wicklow, Ireland). The wine had a low concentration of phenolic substances, as anthocyanin and Total Phenolic Index (TPI) quantified according to Ribéreau-Gayon and Stonestreet (1965) and Ribéreau-Gayon (1970) were 309 and 36 mg/L, respectively. Free and total sulfur dioxide concentration determined according to the iodometric method (Organisation Internationale de la Vigne et du Vin 2009) was 22 and 48 mg/L, respectively.

Tasting trials suggested no peppery notes in the base wine which was described as 'fruity'. The rotundone concentration in the base wine was 9 ng/L determined by the method of Mattivi, et al. (2011). To our knowledge, this level of concentration is the lowest ever detected for a Duras red wine made using the traditional maceration technique (i.e. without pre-ferment removal of skin) from grapes collected at least 37 days after mid-veraison. In fact for this cultivar, a concentration ranging from 18 (Geffroy, et al. 2014) to 341 ng/L – an unexpected high concentration found in wine made from PM infected grapes (Geffroy et al. 2015b) – has been previously reported. Such variation combined with a pilot study using a small group of tasters allowed us to determine the amount of rotundone to add to the base wine. Food grade rotundone (purity > 99%) provided by Firmenich (Geneva, Switzerland) was used to spike the wine at the rate of 25, 50, 100, 200 or 400 ng/L. The spiked wines and an unspiked Control wine were prepared 24 h before the tasting. A 200 ng/L rotundone solution was also prepared for triangle tests with the aim to identify anosmic respondents among the panellists.

Samples were coded with three-digit codes and a constant volume of 15 mL was poured in clear wine-tasting glasses at 18°C. After the tasting, determination of rotundone (Mattivi et al. 2011) confirmed that the concentration of rotundone added were appropriate with a recovery rate of 86 to 97%. The study was organised in Toulouse, south-west of France, in a professional tasting room with booths.

Procedures

The procedure for CRT determination was described by Prescott, et al. (2005) and is based on replicate series of five paired comparison tests, one for each rotundone concentration. Each pair consisted of a sample of the base wine alone and a sample of the base wine with added rotundone. Presentation order was randomised, and each pair was presented in order of ascending concentration. Panellists were asked to proceed with an olfactory assessment, to place the whole sample in their mouth, to move it around for a few seconds and to expectorate it. Then they had to indicate on a sheet which coded sample of the pair they preferred. Panellists received a new pair of samples every 5 min.

At the end of the session and just before to completing the post-experiment questionnaire, anosmic respondents were identified using a triangle test in which one the three coded samples contained a 200 ng/L rotundone solution and the other two samples water alone. The position of the rotundone sample within each test was randomised across each series. Those who were not able to identify the different sample after the olfactory and taste assessment were considered as anosmic respondents.

Data treatment

The binomial distribution for paired comparison test (Roessler, et al. 1978) was used to determine when a wine was significantly rejected. Statistical analyses including principal component analysis (PCA), agglomerative hierarchical clustering (AHC), Chi-square test were conducted with Xlstat software (Addinsoft, Paris, France). The Kolmogorov–Smirnov test was used to assess whether preference for rotundone differed according to gender, knowledge and self-reported liking of peppery notes. For age, socio-economic classification, frequency of consuming, average price spent on bottle and level of knowledge the Kruskal–Wallis test was used. In case of statistical significant results at the 5% threshold for the Kruskal–Wallis test, post-hoc comparisons were performed using Conover–Iman test.

Results

Nineteen anosmic respondents which represents 31% of the whole panel were identified. As they are likely to add noise to the data, they were considered separately. A Chi-square test showed that the proportion of women within the non-anosmic (46.5%) and anosmic populations (42.0%) were equivalent (P = 0.748). The same statistical method revealed that the proportion of panellists over 55 was significantly larger (P = 0.028) within the anosmic population.

Figures 1 and 2 show, respectively, the proportion of non-anosmic and of anosmic panellists who preferred the control wine at each concentration of rotundone tested. Surprisingly, the wine spiked at 25 ng/L was significantly dispreferred by anosmic panellists using the 5% significance criterion (0.79) for paired preference tests (n= 19). For non-anosmic respondents, no CRT was determined which suggests the existence of several groups of consumer behaviour among this population. To determine these clusters, preference data were first treated with PCA. The data set consisted of matrix of order 43 × 5, where the rows represented the 43 non-anosmic panellists and columns represented the preferred rotundone concentration for each of the five paired comparison tests.

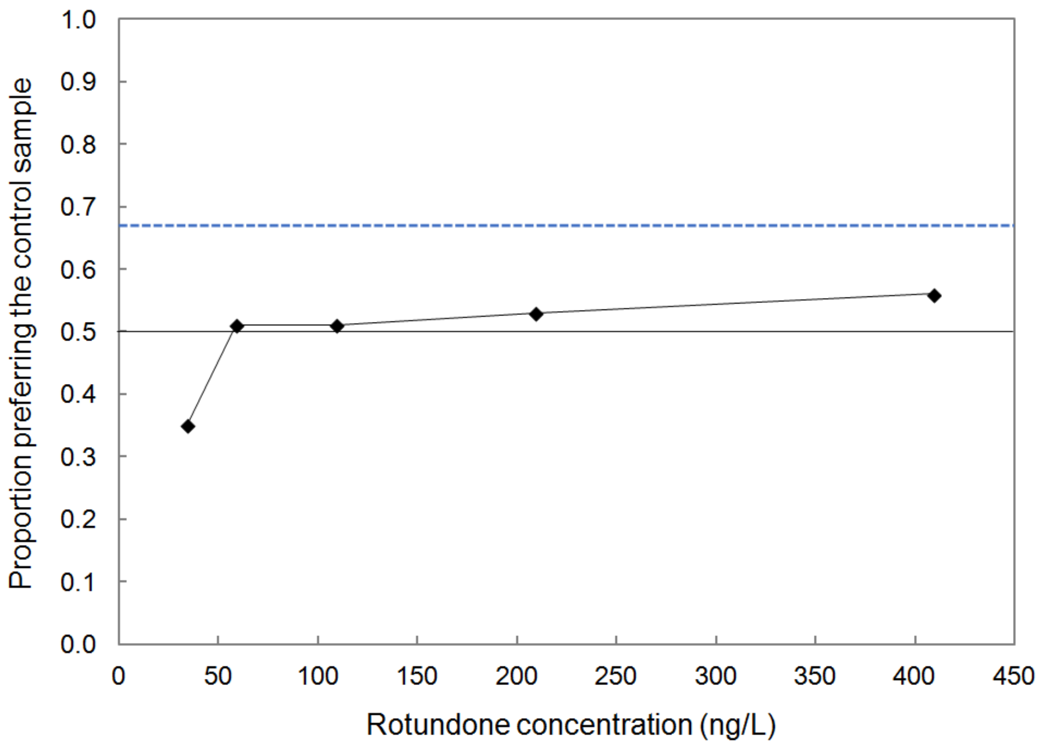

Figure 1. Proportion of non-anosmic panellists preferring the Control sample (*n* = 43). No preference (——) and the Control is significantly preferred at the 5% threshold using paired comparison tests (– – –) are indicated.

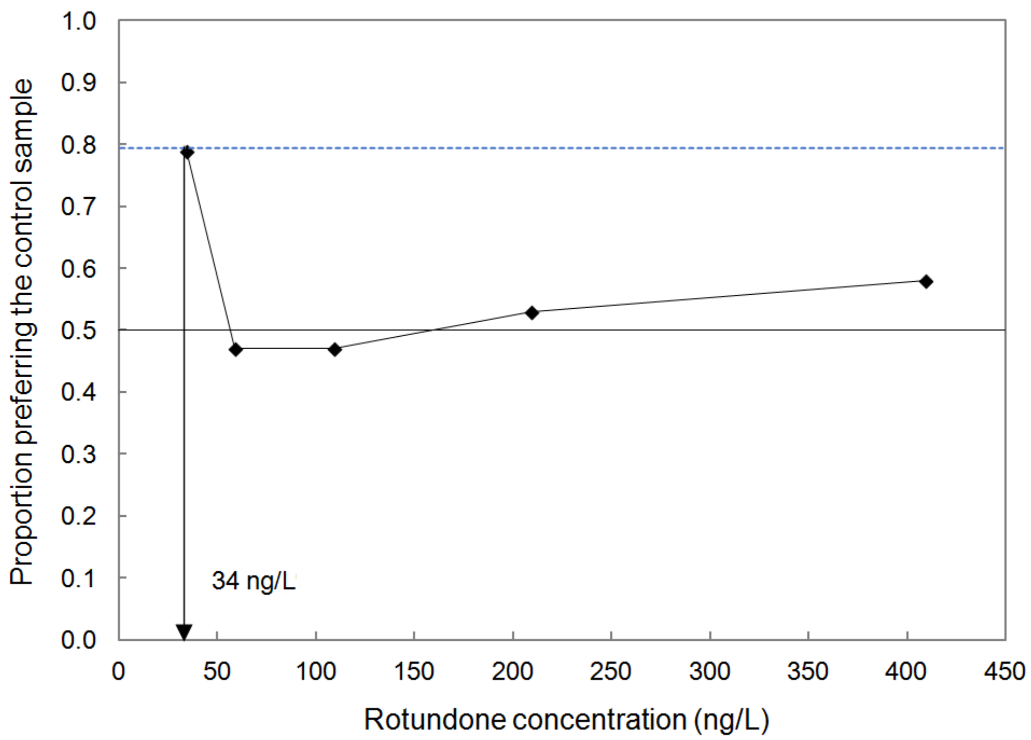

Figure 2. Proportion of anosmic panellists preferring the Control sample (*n*= 19). No preference (——) and the Control is significantly preferred at the 5% threshold using paired comparison tests (– – –) are indicated.

Then an AHC (Figure 3) was performed on the PCA coordinates. The analysis was made using the Euclidian distance and with Ward's method as aggregation criterion. Figure 4 illustrates the proportion of panellists from the three identified clusters who preferred the unspiked wine. The proportion of consumers in each cluster by demographic, consumption habit and wine knowledge categories is presented in Table 1.

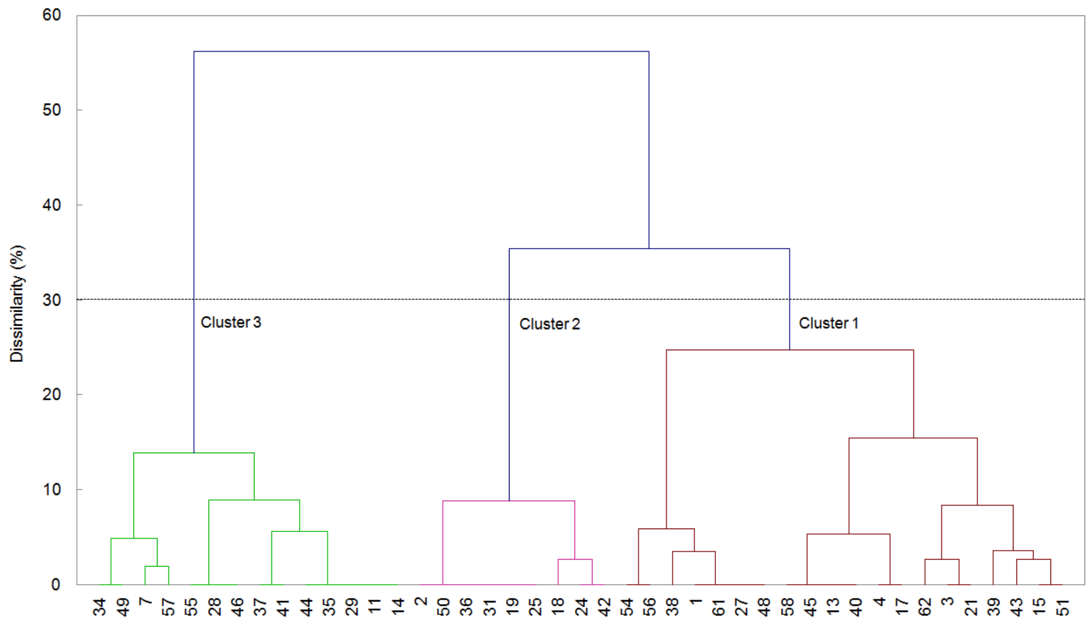

Figure 3. Dendrogram of agglomerative hierarchical clustering showing the existence of three groups of non-anosmic consumers. Numbers on the x-axis refer to identification numbers from 1 to 62 provided to each panellist at the beginning of the study.

It appears that consumers from cluster 1 (*n* = 20) significantly prefer wines with a moderate concentration of rotundone (less than 46 ng/L) and reject wines with ahigh concentration (more than 309 ng/L). This consumer group is mainly composed of males (65%) and panellists over 35 (55%). It presents a substantial proportion of managers (40%) and a good wine knowledge as 60% of the panellists are 'experts' or 'knowledgeable wine drinkers'. Most of them (65%) declared liking peppery notes.

Those from cluster 2 (*n* = 9) significantly prefer the unspiked wine except when rotundone concentration exceeds 275 ng/L in which case neither the control wine nor the spiked wine are preferred. This group has more female (67%), is composed of young panellists as 66% of them are less than 35. These consumers drink wine more occasionally (no more than once per week for 77% of them) and have a basic wine knowledge (78%). Most of them declared disliking peppery notes in wine (66%).

Consumers from the third cluster (*N* = 14) significantly prefer peppery wines when rotundone concentration exceeds 94 ng/L. They are mainly managers (50%), drink wine several times per week (64%) and declare appreciating peppery notes in wine (93%).

Non-parametric statistical tests showed that the age of panellists had an impact on the appreciation of wines for two levels of rotundone concentration, 100 ng/L and 400 ng/L (Table 2). For the wine spiked at 100 ng/L, consumers' preference differed significantly between the youngest panellists (18-24) who preferred the control wine and the oldest ones (>55) who appreciate the spiked wine. For the highest level of rotundone concentration tested in our study, differences were observed between the 25 to 34 and 35 to 44 age categories. The first category preferred the complemented wine while the second one the unspiked wine.

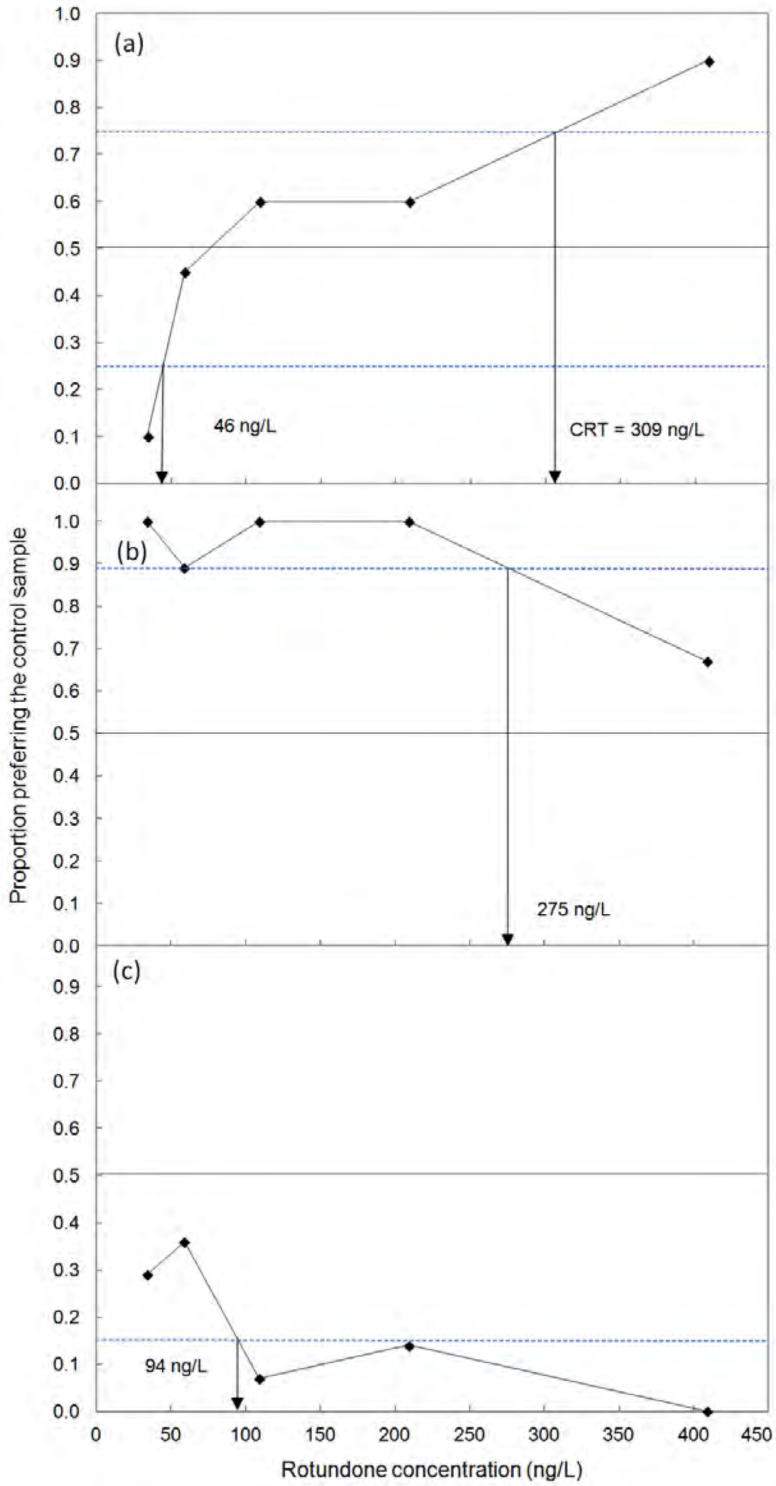

Figure 4. Proportion of panellists preferring the control sample: (a) for the first cluster of non-anosmic consumers (*n*= 20); (b) for the second cluster of non-anosmic consumers (*n*= 9); and (c) for the third cluster of non-anosmic consumers (*n*= 14). No preference (——) and the Control is significantly preferred or dispreferred at the 5% threshold using paired comparison tests (– – –) are indicated. CRT, consumer rejection threshold.

Table 1. The proportion of consumers in each cluster of non-anosmic panellists by gender, age, socio-economic classification, frequency of consuming, average price spent on bottle, levels of knowledge in wine and peppery notes and self-declared appreciation of peppery notes.

Category		**Cluster *n* = 20**	**Cluster *n* = 9**	**Cluster *n* = 14**
Gender	Male (*n* = 23)	65	33	50
	Female (*n* = 20)	35	67	50
Age (years)	18–24 (*n* = 11)	30	44	7
	25–34 (*n* = 11)	15	22	43
	35–44 (*n* = 9)	30	22	7
	45–54 (n = 8)	15	12	29
	>55 (*n* = 4)	10	0	14
Socio-economic classification	Farmers (*n* = 1)	5	0	0
	Middle-level occupations (*n* = 1)	0	0	7
	Managers (*n* = 17)	40	11	50
	Service, sales and support workers (*n*	5	11	0
	Employees (*n* = 15)	30	55	29
	Students and unemployed workers (*n*	20	23	14
Frequency of consuming	Once a day (*n* = 6)	15	11	14
	3–4 times a week (*n* = 5)	20	22	50
	Once a week (*n* = 19)	55	22	29
	Twice a month (*n* = 5)	10	45	7
	Once a month (*n* = 0)	0	0	0
Average price spent on bottle	<€3 (*n* = 0)	0	0	0
	€3 to €5 (*n* = 6)	5	22	21
	€5 to €10 (*n* = 29)	75	67	57
	>€10 (*n* = 8)	20	11	22
Level of wine knowledge	Interested in wines (*n* = 23)	40	78	57
	Knowledgeable wine drinkers (*n* = 13)	35	11	36
	Experts (*n* = 7)	25	11	7
Have you heard of peppery notes in wine?	Yes (*n* = 36)	85	100	71
	No (*n* = 7)	15	0	29
Do you appreciate peppery notes in wine?	Yes (*n* = 30)	65	44	93
	No (*n* = 13)	35	66	7

Table 2. Comparison of preference within the non-anosmic population for each rotundone concentration, for gender, age, socio-economic classification, frequency of consuming, average price spent on bottle, level of knowledge in wine and self-reported liking of peppery notes.

Category	**Significance according to rotundone concentration**				
	34 ng/L	**59 ng/L**	**109 ng/L**	**209 ng/L**	**409 ng/L**
Gender†	0.097	0.999	1.00	0.70	1.00
Age‡	0.564	0.342	0.047	0.115	0.044
Socio-economic classification‡	0.121	0.725	0.201	0.427	0.550
Frequency of consuming‡	0.499	0.101	0.100	0.167	0.166
Average price spent on bottle‡	0.795	0.491	0.681	0.537	0.415
Level of wine knowledge‡	0.156	0.555	0.395	0.584	0.214
Familiarity with peppery notes in wine†	0.261	1.00	1.00	1.00	0.564
Self-report liking of peppery notes‡	0.514	0.988	0.169	0.258	0.377

†The Kolmogorov–Smirnoff (2-tailed) test was performed. ‡The Kruskall–Wallis test was performed.

Discussion and conclusion

The proportion of anosmic respondents within our population (31%) appears to be higher than that observed in Australia (20–25%). Specific anosmia, which refers to the reduced ability (10–1000 times) of an individual to detect a particular odorant, has been widely documented. For androsterone, one of the most notable and studied example, differences in the perception were found between populations from continental Europe, Asia and Australia (Wysocki and Gilbert 1989). These observations corroborate our hypothesis that the rate of specific anosmia to rotundone might differ between French and Australian populations. Our findings also indicate that older panellists (>55) are less likely to detect rotundone. In a large-scale study, it was noticed that a steep decline in detection began more abruptly in the fifth decade for aroma compounds (i.e. androstenone and galaxolide) with previously documented specific anosmia (Wysocki and Gilbert 1989).

The fact that the lowest concentration of rotundone addition (25 ng/L) induced, within the anosmic population, a stimulus leading to the rejection of the spiked sample deserves further comment. Among anosmic panellists who preferred the control wine for this series (n = 15), 47 and 27% of them indicated, in the space available for free comments, that they found the complemented wine less fruity and more acidic, respectively. It is well known that most odorants stimulate the trigeminal system and it has been demonstrated that anosmic respondents were capable of detecting trigeminal sensations (Doty, et al. 1978). Even if our observations suggest that rotundone might induce a trigeminal stimulus, reasons why no sensation was perceived by anosmic panellists at higher rotundone concentration remain unclear. It was recently showed that 2,4,6-trichloroanisole (TCA) attenuated olfactory transduction even at extremely low concentration without evoking odorant responses (Takeuchi et al. 2013). Suppression by TCA was correlated with the lipophilicity of TCA and appeared to be mediated by a partitioning of the compound into the lipid bilayer of plasma membranes. Rotundone is known to be a lipophilic compound (Caputi, et al. 2011) and such molecular mechanisms of flavour reduction might also be involved. As these mechanisms are concentration-dependant (Takeuchi et al. 2013), we might suppose that rotundone concentration in the spiked wine from the first pair was not sufficient to suppress the olfactory signal transduction while for the four following pairs of wines with higher rotundone concentration, the suppression was effective. Molecular mechanisms involved in specific anosmia to rotundone seem to be complex and would deserve further studies.

When focusing on non-anosmics respondents, we were not able to determine any concentration of rotundone from which peppery notes become undesirable in Duras red wine. This illustrates the complexity of the response to this aroma compound and the existence of several clusters of consumer behaviour. Our data indicate that rotundone, unless it is present at unusually high concentration (>309 ng/L), is globally positively perceived or in a neutral manner by two of the three clusters representing 79% of the non-anosmic population. The second cluster, much smaller and mainly composed of young consumers with less experience in wine, preferred the fruity Control wine. This opposition of appreciation between young (18–24) and older (>55) consumers is particularly marked for the 100 ng/L series, a common level of rotundone concentration found in Duras wine. In accordance with previous studies conducted on Gamay wines (Geffroy, et al. 2016), these findings suggest that winemakers should promote moderate to significant levels of rotundone in their Duras wine unless the product is specifically targeted to young consumers.

Interestingly, the real appreciation of peppery notes for each cluster, assessed through our study, was in accordance with consumers' self-report liking. This coherence tends to indicate that panellists had a good overall knowledge in wine and were able to analyse their olfactory sensation. Our study was conducted in Toulouse, south-west of France. This place is favourable notably because of its climatic conditions (Geffroy et al. 2014, 2016b) to the expression of peppery notes in wines. The post-experiment questionnaire revealed that most panellists had previously heard of peppery notes in wine. Unfamiliarity is susceptible to impact the appreciation of a given flavour (Saliba et al. 2009) and participants less familiar with peppery notes in wine could have rejected spiked samples. It is not possible to exclude that, in other French cities or countries, we might have been able to calculate a CRT for rotundone for the non-anosmic population.

Our data are, of course, valid for a particular red wine made from the Duras cultivar. It is important to mention that the base wine made especially for the study is likely the Duras wine with the lowest rotundone concentration ever produced. To generalise our results, we should have measured the detection threshold and conducted the CRT experiment in several wines. During the pre-experiment pilot study, the wine spiked at 25 ng/L had slightly spicier and less fruity notes in comparison with that of the control which is in accordance with the authors' experience with this cultivar. Therefore, the hypothesis that our results might be transposable to other grape cultivars for which rotundone makes an important contribution from the same level of concentration (30 to 35 ng/L) cannot be completely discarded.

If the number of consumers used in our study (62) exceeds the panel size of previous research work on CRT that varied from 35 (Campo et al. 2012) to 58 (Prescott et al.), it is important to mention the relative weakness of our clustering data. According to MacFie (2007), 40 responses per cluster are a minimum to make meaningful interpretations and we cannot completely exclude the likelihood of a significant result by chance especially for the smallest group (cluster 2). In addition, the clustering may have simply detected artefacts such as those individuals who have a specific sample presentation order, or those individuals susceptible to fatigue.

Our study provides winemakers from the Protected Designation of Origin Gaillac with key parameters to adapt their products to consumer profiles. It also opens new fields of investigation into the mechanisms involved in specific anosmia to rotundone.

Acknowledgements

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Chapitre 3 :

Facteurs environnementaux et viticoles influençant la concentration des vins en rotundone

1 Variabilité clonale

Si tous les **génotypes** ne possèdent pas l'aptitude à synthétiser la rotundone, des différences de concentration ont préalablement été décrites entre des **clones** d'une même variété (Caputi, et al. 2011; Siebert et Solomon 2011). Cependant, le manque de robustesse des dispositifs expérimentaux utilisés dans ces études et le fait que les observations soient réalisées sur un seul millésime rendaient difficiles toute conclusion définitive. Ainsi, nous avons cherché à évaluer la variabilité clonale de richesse en rotundone chez le Duras N, au cours de deux millésimes et sur un vignoble expérimental composé de 4 rangs, chaque rang planté avec l'un des 4 clones certifiés de ce cépage (554, 555, 627 et 654). La pureté du matériel végétal a pu être garantie puisque les porte-greffes et les greffons provenaient directement du conservatoire initial du Domaine de l'Espiguette. Depuis sa plantation, la parcelle considérée homogène et utilisée pour la pré-multiplication de la vigne, a fait l'objet d'un contrôle sanitaire rigoureux. L'homogénéité du dispositif a également été vérifiée par des mesures de circonférences de tronc - un indicateur corrélé à l'hétérogénéité du sol selon Tisseyre, et al. (2005), de statut hydrique et de vigueur, des paramètres dépendants du sol et du porte-greffe.

Nous avons ainsi identifié des **différences clonales** de sensibilité à l'oïdium et **de richesse en rotundone** chez le Duras N (Article 3). Des concentrations significativement supérieures ont été relevées dans les vins élaborés à partir des clones **654** et **554** en comparaison avec ceux provenant du clone **555**. Malgré ces différences, le clone n'a pas été identifié, dans le cadre d'une autre étude (Article 4), comme une variable clé permettant de modéliser la concentration en rotundone sur plusieurs sites à partir de variables quantitatives et qualitatives.

2. Facteurs environnementaux

2.1 Environnement abiotique

Par sa nature sesquiterpénique, la synthèse de la rotundone pourrait être régulée par la température, la lumière, la contrainte hydrique et les stress biotiques (Duhl 2008). Dans le cadre d'une étude menée en 2013 et en 2014 sur 10 vignobles de Duras N (AOP Gaillac et IGP Côtes du Tarn), nous avons démontré que le mésoclimat, à travers le **cumul de précipitation**, l'**indice d'Huglin**, l'**irradiation moyenne quotidienne,** et le **nombre d'heures d'ensoleillement** au cours de la maturation des raisins étaient des paramètres clés permettant d'expliquer les différences de concentration en rotundone entre les sites et entre les millésimes (Article 4).

Parmi les variables citées précédemment, celles liées aux **précipitations** ou au **statut hydrique** de la plante au cours de la maturation des raisins, paraissent déterminantes pour discriminer la concentration en rotundone des vins en fonction des millésimes (Article 1, Article 5, Article 6) et au niveau intra-parcellaire (Article 5). Au sein d'une même parcelle, nous avons pu établir une corrélation entre la concentration en rotundone et le **$\delta^{13}C$** (R2 = 0.76 et R2 = 0.74 en 2012) (Article 5), un indicateur du statut hydrique du végétal au cours sur la période véraison-récolte. Le rôle de la **température,** notamment au cours de la maturation, apparaît moins fondamental. En effet, les concentrations en rotundone des vins produits lors de millésimes chauds et arrosés sont généralement supérieures à celles observées lors de millésimes plus frais et plus secs (Article 5, Article 6).

2.2 Environnement biotique

Nos travaux ont également permis de mettre en évidence une corrélation négative entre l'**acide gluconique**, un métabolite secondaire de *Botrytis cinerea*, et la concentration en rotundone des vins (R2 = 0.69) (Article 4). Ces observations suggèrent que ce champignon saprophyte ou sa polyphénol oxydase, seraient capables de dégrader la molécule. Nous avons aussi pu démontrer qu'*Erysiphe necator*, le champignon responsable de l'oïdium de la vigne, induisait une réponse différente chez la plante (Article 3). Une corrélation positive a pu être établie entre l'**intensité des dégâts d'oïdium** sur grappes et la **concentration en rotundone** (R2 = 0.58). Cette relation laisse supposer que le champignon est capable de stimuler la production de rotundone. Il est important de mentionner que les concentrations mesurées dans les vins élaborés à partir de grappes atteintes d'oïdium, supérieures à 300 ng/L dans la plupart des cas, figurent parmi les plus élevées reportées dans la littérature. Les **attaques d'herbivores** semblent être sans effet sur la synthèse de la molécule puisque les vins élaborés à partir de grappes infectées par *Drosophila melanogaster* et/ou *Drosophila suzukii* ne sont pas davantage pourvus en rotundone que des vins provenant de parcelles saines environnantes. (Article 7). Ces observations sont cohérentes avec d'autres résultats issus de nos recherches mettant en évidence qu'une pulvérisation d'**acide jasmonique** orientée sur les raisins, une phytohormone impliquée dans les mécanisme de défense de la plante notamment lors de l'attaque d'insectes, n'avait aucun impact sur la rotundone (Article 5).

3 Pratiques viticoles

Même si le mésoclimat apparaît comme un facteur clé, la concentration en rotundone est susceptible d'être impactée comme d'autres composés aromatiques par les **pratiques viticoles** (Alem, et al. 2018). Ainsi, la mise en œuvre de techniques culturales spécifiques pourrait permettre aux vignerons, en fonction de leurs objectifs, d'orienter le profil sensoriel de leurs vins en favorisant ou en limitant l'accumulation de la rotundone.

Nous avons démontré que la **date de récolte** possédait un fort impact sur la concentration des vins en rotundone et que la molécule s'accumulait plutôt tardivement. À Gaillac, la concentration en rotundone augmente avec la maturation et atteint un plateau 44 jours après la mi-véraison (Article 5). A Marcillac, dans un contexte climatique plus frais, nous avons mis en évidence que le fait de différer la récolte de 7 jours engendrait un gain dans les vins supérieur à 100 % (Article 6).

L'**éclaircissage** des grappes, réalisé à une intensité de 40 % à la véraison, n'a aucun impact sur la teneur des vins en rotundone (Article 5). Ces observations laissent à penser que l'accumulation de la molécule est indépendante des relations de type source – puits et qu'elle est produite *in situ* dans les raisins. Ces constations sont en accord avec d'autres résultats issus de nos recherches mettant en avant que l'arrêt du flux de sève *via* le sectionnement de la branche à fruits ne perturbe pas l'accumulation de la rotundone (Article 7).

L'**effeuillage**, une opération en vert consistant à éliminer les feuilles de la zone fructifère, possède un impact variable sur la rotundone. Nous avons montré que cette pratique, réalisée les deux faces du rang à la véraison, pénalisait fortement la concentration en rotundone des vins (Article 5). L'effeuillage mis en œuvre 10 jours après la nouaison sur la face du rang exposé côté soleil levant, n'a eu aucun impact (Article 6). Cette pratique a même provoqué, en tendance, une légère augmentation de la rotundone dans les vins. L'élimination des rameaux latéraux ou entre-cœurs (**échardage**) est quant à lui sans effet sur la rotundone (Article 6).

L’apport d’eau avant la véraison via l’**irrigation** induit de manière systématique une augmentation de la concentration en rotundone dans les vins (Article 5, Article 7). Ces observations sont cohérentes avec nos conclusions précédentes (cf 2.2.1) soulignant le rôle déterminant du cumul de précipitations et du statut hydrique de la plante. Etant donné que l’absence de contrainte hydrique est défavorable à l’accumulation des composés phénoliques du raisin (Ojeda, et al. 2002), il pourrait s’avérer problématique d’élaborer des vins riches en rotundone et en composés phénoliques. Nous avons montré que le **Passerillage Eclaircissage sur Souche (PES)**, une technique consistant à sectionner 3 semaines avant la récolte la branche à fruits sur une vigne conduite en Guyot, possédait un impact limité sur la rotundone et permettait d’enrichir le vin en polyphénols par concentration (Article 7). Ces résultats nous ont permis de construire un système viticole combinant irrigation, pour favoriser la production de la rotundone, puis PES, pour réduire les effets dépréciatifs de l’irrigation. Ce système s’est montré particulièrement performant puisqu’il a engendré un gain significatif en rotundone, en sucres, en anthocyanes et en polyphénols totaux (Article 7).

Article 3:

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Certified clone and powdery mildew impact rotundone in red wine from *Vitis vinifera* L. cv. Duras N

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Résumé : Cette étude a permis de mettre en évidence des différences de richesse en rotundone entre les 4 clones certifiés de Duras N (554, 555, 627 et 654). Lors du deuxième millésime d'étude, une corrélation positive a été établie entre l'intensité des dégâts d'oïdium (*Erysiphe necator*) observées sur grappes et la concentration en rotundone des vins. Ces constations suggérant que la molécule pourrait être produite en réponse à un stress biotique, pourrait permettre d'expliquer les niveaux de concentration en rotundone particulièrement élevés observés dans les vins de ce millésime.

CERTIFIED CLONE AND POWDERY MILDEW IMPACT ROTUNDONE IN RED WINE FROM *VITIS VINIFERA* L. CV. DURAS N

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Abstract

Aim: Few recent studies have been investigating the effect of clone on aroma compounds. The aim of this research work was to study the impact of certified clones from *Vitis vinifera* L. cv. Duras N on grape quality and rotundone, a sesquiterpene responsible for peppery aroma which has been reported recently in red wines made from this cultivar.

Methods and results: The experimental site consisted of four consecutive rows, each row planted with one of the four certified clones of Duras N (554, 555, 627 and 654). For each clone, measurements were replicated on three experimental units per row. Each experimental unit consisted of twelve continuous vines. Rotundone concentration was measured in wines prepared by microvinification techniques (1-L Erlenmeyer flasks). For both vintages of study, rotundone concentrations were significantly higher in wines from clones 554 and 654 in comparison with clone 555, while clone 627 showed an intermediate level. In 2014, differences in powdery mildew (PM) severity on clusters were identified between the four clones and a positive logarithmic correlation ($r^2 = 0.58$) was reported on the experimental site between rotundone in wines and PM severity on grapes.

Conclusion: Our results found differences in rotundone concentrations in wines made from the four Duras N certified clones. The results also suggested that grapevine defence response to PM could enhance rotundone production in berries. Clonal differences in susceptibility to biotic stress, such as PM, might explain the differences observed in rotundone concentrations between the four studied clones.

Significance and impact of the study: Our results may assist grape growers to produce high quality wines with a desired aroma attribute made from Duras N. One should consider planting clone 554 in order to promote high levels of rotundone in wines made from this cultivar.

Key words: rotundone, Duras N, certified clone, powdery mildew, natural defence

Résumé

Objectif : Peu d'études récentes ont étudié l'effet du clone sur les composés aromatiques. L'objectif de ce travail de recherche était d'évaluer l'impact des clones certifiés de *Vitis vinifera* L. cv. Duras N sur la qualité des raisins et la rotundone, un sesquiterpène responsable des arômes poivrés identifié récemment dans les vins élaborés à partir de ce cépage.

Méthodes et résultats : Le site expérimental se compose de quatre rangs consécutifs, chacun des rangs étant planté avec l'un des quatre clones certifiés de Duras N (554, 555, 627 et 654). Pour chacun des clones, les mesures ont été répétées sur trois placettes expérimentales par rang, chacune d'entre elle se composant de douze pieds consécutifs. La concentration en rotundone a été mesurée dans des vins élaborés en condition de microvinification (Erlenmeyer d'1 L). Pour les deux millésimes d'étude, les concentrations en rotundone ont été significativement supérieures dans les vins des clones 554 et 654 en comparaison avec le clone 555 alors que le clone 627 a présenté un niveau intermédiaire. En 2014, des différences d'intensité de dégâts d'oïdium ont été mises en évidence sur grappes entre les quatre clones et une corrélation logarithmique positive ($r^2 = 0.58$) a pu être établie sur le site expérimental entre la rotundone dans les vins et l'intensité de ces dégâts.

Conclusion : Nos résultats ont permis de mettre en évidence des différences de concentrations en rotundone dans les vins élaborés à partir des quatre clones certifiés de Duras N. Les résultats suggèrent également que les réactions de défense de la vigne vis-à-vis de l'oïdium peuvent induire la production de rotundone dans la baie. Des différences clonales de sensibilité à un stress biotique, comme l'oïdium, pourrait permettre d'expliquer les différences de concentrations en rotundone observées entre les quatre clones étudiés.

Signification et impact de l'étude : Nos résultats sont susceptibles d'aider les producteurs à élaborer des vins de Duras N de qualité possédant des notes aromatiques recherchées. Ces derniers ont intérêt à planter le clone 554 s'ils souhaitent favoriser des concentrations en rotundone élevées dans les vins de ce cépage.

Mots clés : rotundone, Duras N, clone certifié, oïdium, défense naturelle

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INTRODUCTION

According to the definition of the Office International de la Vigne et du Vin (OIV), "one clone is the certified vegetative descent of one vine chosen for its identity, its phenotypic characteristics and its sanitary condition". One clone may have been selected deliberately from a grapevine that has demonstrated desirable traits. That is why differences in phenotypes are often found within certified clones of a given grape variety. Clones can differ in morphological characteristics (leaf, cluster and berry shapes) which can lead to different levels of susceptibility to grape disease such as *Botrytis cinerea*, agronomical behaviour (earliness of ripening, vigour, fertility, sensitivity to flower abortion, number of berries per cluster, cluster weight, grape yield), and berry traits (sugar, acidity, phenolic and aroma contents at harvest). Despite large progress in the chemistry of wine aroma during the last two decades, few recent studies have been investigating the impact of clone on aroma compounds or using aroma analysis as a tool for selecting new clones.

Most of the studies on this topic were conducted on white aromatic cultivars with a focus on free and bound monoterpene contents of the investigated clones. Differences were identified within clones of Gewürztraminer and Weisser Riesling (Marais and Rapp, 1991), Chardonnay (McCarthy, 1988a; Versini *et al.*, 1988) and Muscat à petits grains (McCarthy, 1988b). Comparing Merlot noir clone wines, Kotseridis *et al.* (1998) reported higher levels of 3-isobutyl-2-methoxypyrazine in the wine of French certified clone 182. Another study investigated the aroma of wines from seven clones of Monastrell grapes that allowed some grouping of the clones (Gómez-Plaza *et al.*, 1999).

More recently in Shiraz grapes, Siebert and Solomon (2011) found a higher level of rotundone in the 2626 clone in comparison with the 1127. This key aroma compound, identified for the first time in 2008 in Shiraz wines (Wood *et al.*, 2008), was also reported recently by Geffroy *et al.* (2014) in Duras N wines, a grape variety grown in the South West of France.

Duras N is one of the main red grape cultivars in the Protected Designation of Origin (PDO) Gaillac. Its name is derived from "dur" (meaning "hard"), which refers to the hardness of its shoots. It was first mentioned in Gaillac in a document from 1484, and seems to have been continually cultivated since then in the area. Several hypotheses were advanced to explain the deep origin of this cultivar, considering that Gaillac – one of the oldest vineyards in France – was founded in the second century BC (Viala and Vermorel, 1902; Lavignac, 2001). The presence in forests and riversides around the Gaillac area of many relict populations of *Vitis vinifera* ssp. *sylvestris*, locally called "lambrusques", made some authors think that Duras could be an offspring of this wild vine. Other writers supposed Duras to be the Duracina variety cited by the Latin authors Cato the Elder, Pliny the Elder and Columella (Lavignac, 2001). But no evidence or clue could be provided to confirm these suppositions as no genetic relationships were found between local varieties and wild vines in the area (Olivier Yobrégat, unpubl. data, 2014), and a simple similarity between two cultivar names does not constitute an evidence to pretend they are identical, especially with more than 1 500 years of difference. Recently, Lacombe *et al.* (2013) discovered that Duras N was an offspring of two other ancient cultivars from the North East of France, Savagnin B and Tressot N.

Four different clones of this cultivar, almost exclusively represented in the PDO Gaillac, are presently available. The clones are virus-tested, certified and designated under the numbers 554, 555, 627 and 654. Clones 554 and 555 were the first to be certified in 1978 (MAP, 2007), were the first to be planted for multiplication and are currently the most profuse. In one study (Daniel Goulard, unpubl. data, 1993), the four clones were described as homogenous for pruning wood weight, grape yield and analytical characteristics of the grapes at harvest. However, the intensity of rotundone could vary between them. Therefore, a two-year study, commenced in 2013, was conducted in order to evaluate the rotundone concentration in Duras N wines made from the four certified clones.

MATERIALS AND METHODS

1. Experimental site and design

The experimental vineyard, located in the Gaillac vineyard (lat. 43° 50' 32" N; long. 01° 51' 7" E) consisted of four consecutive rows, each row planted with one of the four certified clones of Duras N. Orientation of the vine rows was south-west to north-east with the following sequence: 554, 555, 627 and 654. All measurements were replicated three times on experimental units of 28.5 m^2 consisting of twelve continuous vines. The plot was flat and typical of the area with 2.20 m x 1 m row spacing. It was planted in 1999 with Duras N grapevines grafted onto 140 Ruggeri rootstock (clone 265). Plant materials (rootstocks and scions) came directly from the initial repository of selected clones (IFV Domaine de l'Espiguette, France) where permanent sanitary controls are performed and traceability information is maintained. Vines were trained with vertical shoot positioning (VSP) associated with Cordon de Royat pruning system. The soil was managed by chemical weed control under the vines, and by grass cover inter-row.

2. Description of the clones and sampling

The official description of the agronomical and technological behaviours of the four certified clones of Duras N is presented in Table I. For the four clones, budburst took place on 10 April in 2013 and 4 April in 2014. Mid-veraison was determined by randomly collecting 100 berries on the experimental site and by counting the number of coloured berries. No differences in mid-veraison date (27 August in 2013 and 12 August in 2014) were observed between the four clones. Due to a delay of 3 weeks in phenology and high risks of *Botrytis cinerea* development in 2013, grape samples were collected on 26 September, which was early in the maturation process and corresponded to 30 days after mid-veraison. In 2014, sample collection took place 40 days after mid-veraison on 19 September. In both vintages, picking dates coincided with commercial harvest of the plot. Each year, twelve grape samples were collected at harvest (three per clone). Each sample consisted of 216 berries from both sides of the row and several parts of the bunch (nine berries per vine on each side of the row). Prior to analysis, the weight of 200 berries was measured. Berries were crushed and 100 mL of must were centrifuged (14 000 × *g* for 6 min) to enable classical laboratory and $\delta^{13}C$ analysis. The remaining amount of must and skin was crushed for 2 min using a blender and was used for phenolics determination. Also, from each experimental unit, 800 g of berries were collected to perform microscale fermentations in a 1-L Erlenmeyer flask according to the protocol proposed by Geffroy *et al.* (2014). After alcoholic fermentation, wines were centrifuged (14 000 × *g* for 6 min), supplemented with sulphite (80 mg/L), bottled and stored at 4°C or below until rotundone analysis. In order to obtain a sufficient amount of juice and wine to carry out the analyses, the samples were only composed of healthy berries.

3. Classical laboratory

Sugar concentration (°Brix) was determined with a digital hand-held Pocket refractometer PAL (Atago, Japan). Titratable acidity was measured according to OIV method (OIV, 2009) and pH measured with a HI 3221 pH meter (Hanna Instruments, France). A Konelab Arena 20 sequential analyser (Thermo Electron Corporation, USA) associated with enzyme kits provided by several suppliers was used to determine amino acids, ammonium (Megazyme, Ireland) and malic acid (Thermo Fisher Scientific, USA). Potassium determination was by flame photometry (Bio Arrow, France) according to OIV method (OIV, 2009) and tartaric acid determination by colorimetric titration (Hill and Caputi, 1970). Anthocyanins and Total Phenolic Index (TPI) were quantified in grapes according to Cayla *et al.* (2002) using an Evolution 100 spectrophotometer (Thermo Electron Corporation, USA). This analytical method for phenolics determination in grapes is based on a1-hour micromaceration in a hydroalcoholic and acid medium. All determinations were carried out in duplicate. The remaining amount of centrifuged must was used for $\delta^{13}C$ determination.

4. Rotundone analysis

Because of quarantine requirements and logistical issues with shipping frozen grapes from France to Australia, rotundone was analysed indirectly in wines fermented under microvinification conditions. For the rotundone, reproducibility tests showed an average coefficient of variation of 5% across the microfermentors. Rotundone in wine was determined by solid phase microextraction-multidimensional gas chromatography-mass spectrometry (Geffroy *et al.*, 2014). The analyses were performed in two different

Table1 - Description of the four certified clones of Duras N in France (MAP, 2007).

Clone number	Breeder	Year of certification	Surface available for multiplication (ha)	Fertility	Cluster weight	Grape yield	Sugar concentration at harvest
554	ENTAV	1978	1.54	Average	Average	Average	Superior
555	ENTAV	1978	0.44	Average	Inferior to average	Average	Superior
627	ENTAV	1979	0.44	Average to superior	Average to superior	Average to superior	Average to superior
654	ENTAV	1980	<0.01	Average to superior	Average to superior	Inferior to average	Inferior to average

years but during the same period of each year to reduce potential variations associated with different post-bottling times.

5. Measurements on vines and evaluation of powdery mildew severity on grapes

At harvest, number of clusters per vine and yield (kg/vine) were monitored for 12 out of 12 vines for each experimental unit by counting the number of clusters and weighing crop loads individually with a Precia Molen C20 K balance (Precia SA, France). Severe powdery mildew (*Erysiphe necator*) infections were recorded on grapes in 2014. Powdery mildew (PM) severity was assessed at harvest by visually estimating the percentage of the cluster area colonized by the pathogen on 100 grape clusters (50 per each side of the vine) within each experimental unit.

6. Weather measurements

In 2013 and 2014, rainfall and air temperature (minima, maxima and mean values) were monitored by a Cim AGRO weather station (Cimel Electronique, France) placed within 200 m of the experimental site. In order to characterize the two vintages, several indices proposed by Tonietto and Carbonneau (2002) were calculated: the Huglin index or heliothermal index over the period of 1 April to 30 September, the cool night index (FNv-r), the mean air temperature (Tv-r), the maximal air temperature (Txv-r), and the thermal amplitude (Av-r) indices over the veraison-harvest period. Cumulative rainfall over the whole calendar year, the budburst-veraison and veraison-harvest periods was also calculated.

7. Homogeneity control of the plot

Since its plantation, the plot has been used for pre-multiplication, has always been virus-tested and judged homogenous especially regarding pruning wood weight and vigour. All ELISA tests carried out in November 2014 were negative for ArMV, GLFV, GLRaV 1, 2 and 3. Recent studies have highlighted large spatial variations in berry and wine rotundone concentrations within a single vineyard (Geffroy *et al.*, 2014; Scarlett *et al.*, 2014). This variability was associated with variation in the land underlying the vineyard and was linked especially with vine water status. Despite its apparent homogeneity, extra controls were carried out on the plot in order to detect any source of variability, especially between the rows, using:

- Trunk circumference (TC): several studies (Tisseyre *et al.*, 2007; Bramley *et al.*, 2011) showed that this index of vine vigour was well correlated to apparent electrical conductivity of the soil (EC_a) and therefore could be used to monitor soil heterogeneity. More recently, studies by Geffroy *et al.* (2015) showed that a link existed between plant architecture (TC) and rotundone, and that TC could be used to approach rotundone spatial distribution. In April 2014 just before budburst, TC was determined for every vine within each experimental site as the average value measured at three different heights: just above the graft; 10 cm under the trellis wire; and half way between the two first points of measurement.

- $\delta^{13}C$ measurement: $\delta^{13}C$ measured on grapes at harvest is an integrative indicator of water deficit experienced by the vine during grape ripening (Van Leeuwen *et al.*, 2009). Geffroy *et al.* (2014) identified good correlations (r^2 = 0.76 in 2011 and r^2 = 0.74 in 2012) between rotundone concentration in wine and $\delta^{13}C$ within the same vineyard. Tin capsules (TIN 6 × 4 mm) were introduced into a 96-well (8 mm) microplate (model 83.1835; Sarstedt, Germany). Five mL of centrifuged grape juice collected at harvest for each experimental unit were introduced into each of the tin capsules using a micropipette. The microplate was then placed in a non-ventilated oven at 60°C for 24 h, sealed and sent to the stable isotope laboratory of the James Hutton Institute (Dundee, Scotland). Samples were analysed for their bulk 13C isotopic composition by standard operation procedures compliant with the International Union of Pure and Applied Chemistry guidelines and recommendations for stable isotope ratio measurements and reporting results thereof (Coplen, 2011). The precision of the $\delta^{13}C$ values obtained was determined by repeat analysis of reference materials. Quality control samples were better than ±0.15%.

- NDVI mapping: in 2014, remotely sensed imagery (airborne multispectral video imagery with a ground resolution of 50 cm) of the plot was obtained (Lamb *et al.*, 2004) at veraison (4 August). This widely used indicator of plant vigour or relative biomass was calculated from the extracted single-pixel or multiple-pixel values using the relationship proposed by Rouse *et al.* (1973).

To our knowledge, for a given cultivar no clonal differences in drought tolerance, vigour or vine architecture (TC) – variables which are impacted by the rootstock – have been previously described in the literature. Therefore, if some differences were to be observed between the clones for these variables, then they are more likely to reflect variations in soil composition within the experimental site.

8. Statistical analysis

Statistical analyses were conducted with Xlstat software (Addinsoft, France). All the analytical data measured both in 2013 and 2014, and rotundone content of the wines were first subjected to a three-way analysis of variance (ANOVA) treatment (vintage × clone × block) with first-order interaction (n = 24; residual degrees of freedom = 6). Because the block effect was never significant and vintage × clone interactions were almost always significant, data excluding rotundone were next subjected for each year to a one-way (clone) ANOVA (n = 12; residual degrees of freedom = 8). Fisher's least significant difference test was used as a post-hoc comparison of means at $P < 0.05$. Other variables which were just determined with repeated measurements during one year of study (i.e. trunk circumference, severity of powdery mildew) were first treated by a two-way ANOVA (clone × block) with first-order interaction. As the block factor was not significant, data were next analysed by a one-way ANOVA. Regression was undertaken with the PM severity data averaged for each experimental unit.

RESULTS AND DISCUSSION

1. Quantitative and qualitative performances of the clones

Results of the three-way ANOVA treatments (Table 2) show that the vintage factor had the strongest overall impact on quantitative and qualitative performances of the clones. As harvest took place respectively 30 days and 40 days after mid-veraison in 2013 and 2014, it is difficult to compare the two vintages from an analytical point of view. No significant block effect or interactions involving that factor were observed on the plot and NDVI spatial pattern (results not shown), trunk circumference and $\delta^{13}C$ measurements did not allow discrimination of the four studied clones. Despite the imperfection of the experimental design, these observations reflect a good homogeneity of the plot and confirm that differences observed are more likely to be due to a real clone effect than to variation in the land underlying the vineyard, especially between the four rows of study.

Vintage × clone interactions were almost always significant. While differences were observed in 2013 between the four clones for most of the measured variables, few analytical data allowed discrimination of the clones in 2014. In 2014, the severity of powdery infections might have brought some perturbation and variability in the data set. In 2013, clone 554 showed better qualitative performances (i.e. higher sugar concentration, TPI and anthocyanins) in comparison with the other clones and especially clone 555. This is not a trivial matter as the two clones that showed the largest amplitude of difference in analytical variables (554 and 555) were the furthest apart in the vineyard. Also, our observations, in terms of grape yield and sugar concentration at harvest, were not particularly in accordance with the information provided by MAP (2007) and shown in Table 1.

Table 2 - Results of the analysis of variance performed on the data measured during the trial.

Parameter	*P*-value					
	Vintage (V)	Clone (C)	Block (B)	V × C	V × B	C × B
200 berry weight	<0.0001	0.152	0.543	0.035	0.593	0.736
Sugar concentration	0.076	0.002	0.764	0.004	0.296	0.115
Total acidity	0.462	0.033	0.203	0.034	0.386	0.556
pH	<0.0001	0.224	0.150	0.025	0.714	0.718
Tartaric acid	<0.0001	0.011	0.806	0.006	0.290	0.160
Malic acid	<0.0001	0.005	0.223	0.001	0.092	0.103
Amino acids	0.005	0.106	0.777	0.046	0.851	0.148
Ammonium	0.559	0.301	0.502	0.323	0.524	0.506
Potassium	<0.0001	0.481	0.069	0.106	0.982	0.218
Total Phenolic Index (TPI)	0.007	0.009	0.397	0.014	0.917	0.534
Anthocyanins	0.006	0.020	0.353	0.011	0.256	0.400
Number of clusters per vine	<0.0001	0.951	0.126	0.218	0.179	0.390
Yield at harvest	<0.0001	0.125	0.217	0.198	0.397	0.148
$\delta^{13}C$	0.980	0.701	0.961	0.969	0.415	0.521
Severity of powdery mildew	-	<0.0001	0.123	-	-	0.075
Trunk circumference	-	0.478	0.652	-	-	0.603
Rotundone concentration	<0.0001	0.049	0.842	0.127	0.597	0.579

Neither the number of clusters per vine nor yields (kg per vine) at harvest were impacted in 2013 and 2014 by clone type. As significant differences in severity of PM on grapes at harvest were observed between the clones in 2014 and PM infection leads to lighter bunch weight, differences in yields could have been expected. The PM infections could have impacted the analytical variables measured on grapes at harvest as previously reported on Chardonnay grapes and wines, which showed an increase in titratable acidity and total phenolics corresponding with increasing severity of PM infection (Stummer *et al.*, 2005). However, this phenomenon was not observed during our study because only healthy berries were collected for laboratory analyses, minimizing the effects of PM. Differences in susceptibility of grapevine cultivars to PM (Doster and Schnathorst, 1985) and in clonal susceptibility to downy mildew (*Plasmopara viticola*) on leaves (Boso *et al.*, 2004) have been previously described. To our knowledge, this is the first time that clonal differences in powdery mildew susceptibility have been observed on grapes. However, as the experimental site consisted of four rows – each one planted with one different clone – and PM infection was only observed in 2014, it is difficult to draw definitive conclusions. Indeed, the differences observed could reflect variations in quality of the phytosanitary treatment targeted against PM in relation with the spraying equipment. However, there was no direct relationship between the sequence of planting of the four clones (554, 555, 627 and 654) and the severity of powdery mildew recorded on grapes in 2014 (555 < 554 < 627 < 654). Differences in canopy density could have affected PM severity between the four clones; for example, canopy management designed to optimize sunlight exposure of grape clusters was shown to have a beneficial impact on powdery mildew (Austin *et al.*, 2011). Nevertheless, NDVI measurements conducted in August 2014 did not show any significant differences between the four rows and consequently between the clones for this vegetation index. As summarized by Schnee (2008) in his doctoral thesis, the difference of susceptibility to PM could have been induced by i) constitutive factors in relation with physical properties or barriers that limits the penetration of the pathogen (i.e. cuticle) or ii) induced factors in relation with defence responses (i.e. phytoalexin). A large variability in severity of powdery mildew was observed within the clones. This can be attributed to the fact that this variable was determined with repeated measurements for each block and that standard deviations refer both to variability within one block and to inter-block variability.

2. Rotundone in wines

Unlike the other variables measured on grapes and discussed previously, no vintage × clone interaction was observed for rotundone. Concentrations of rotundone were significantly higher in wines from clones 554 and 654 in comparison with clone 555, while clone 627 showed an intermediate level (Figure 1). As rotundone was measured indirectly in wines prepared by microvinification techniques (1-L Erlenmeyer flask), differences observed in sugar content of the grapes in 2013, and thus leading to distinct ethanol concentrations in the resulting wines, might have influenced the extraction of the hydrophobic rotundone from the berry skin. However, under the winemaking conditions of this study, the effect of the ethanol differences would be minimal, which is consistent with other research where the impact of ethanol on the extraction of hydrophobic compounds, such as proanthocyanidins, from the berry skin did not exceed a few percent on mature or almost mature grapes (Canals *et al.*, 2005).

Rotundone concentrations observed in 2014 (more than 300 ng/L on average for three of the studied clones) (Figure 1), are among the highest ever reported and the strong vintage effect observed on the experimental site deserves further discussion. Caputi *et al.* (2011) suggested that higher concentrations were most likely to accumulate in grapes in cooler vintages while Geffroy *et al.* (2014) proposed that summer water stress was a more important variable than air temperature in explaining differences in rotundone between seasons. More recently, Zhang *et al.* (2015) showed that berry temperature exceeding 25°C between veraison and harvest negatively affects the rotundone concentration in Shiraz. The 2013 vintage was characterized by a cold spring and regular, large rainfall events over the vine vegetative cycle which induced a delay of 3 weeks at harvest. In 2014, winter was rainy and warm; summer was extremely rainy with 160 mm of rain recorded between mid-July and the end of August; and conditions during maturation were dry and hot. Climatic indices and cumulative rainfall calculated for the two vintages of study allow the two vintages to be compared in more detail (Table 4). The 2013 vintage was cooler, especially during the veraison-harvest period, and less rainy than 2014 whose rainfall events mostly occurred as summer storms, and the two vintages were characterized by a similar water deficit as reflected by $\delta^{13}C$ measurements (Table 3). Consequently, as bunch temperature appeared to be the main determinant to explain the differences in rotundone between the two vintages, larger concentrations were expected in 2013. As

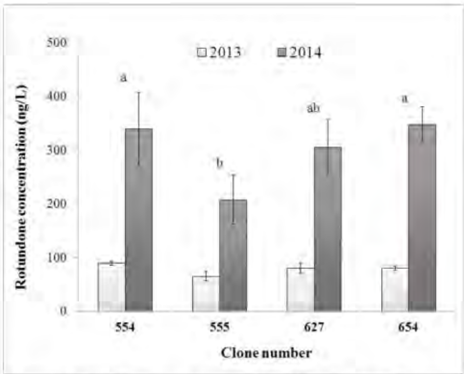

Figure 1 - Rotundone concentration in experimental wines made from the four certified clones of Duras N.
Different letters which refer to both years indicate means significantly different at P< 0.05 by Fisher test.

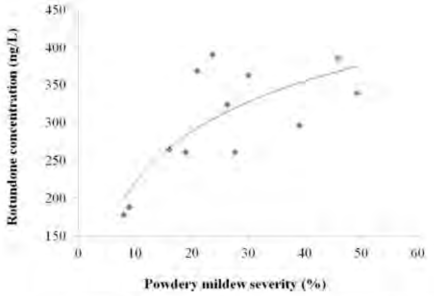

Figure 2. Relationship between rotundone concentration in wines and powdery mildew severity on grapes at harvest in 2014 (n = 12).
Linear model gave y = 96.72 ln(x) – 2.270; r^2 = 0.58; RMSE = 49.2.

grapes were harvested 10 days earlier in 2013 for the clonal study, this difference in lapse of time after mid-veraison must have impacted rotundone concentrations in the resultant wines. Geffroy *et al.* (2014) showed, in the same viticultural context, that rotundone concentration greatly increased between mid-veraison +30 days and mid-veraison +44 days in red wines from Duras N. As a result, differences in rotundone concentrations expected between the vintages for the clonal study should remain small and in no case as large as determined in our study. This suggests that another mechanism(s) is involved.

Host responses to PM have been characterized in various resistant species such as *Vitis labrusca*, *Vitis rupestris* (Doster and Schnathorst, 1985), *Vitis aestivalis* (Fung *et al.*, 2008) and *Vitis pseudoreticulata* (Weng *et al.*, 2014), a wild Chinese species. This last study carried out on leaves showed that infection by PM induced differential expression of 6541 genes with several of them belonging to the defence, salicylic acid (SA) and jasmonic acid categories. One of the few studies which have been investigating the transcriptional response to PM in susceptible *Vitis vinifera* (Fung *et al.*, 2008) showed that endogenous SA levels were lower in *Vitis vinifera* than in *Vitis aestivalis* in the absence of fungus and that SA concentrations increased with PM in *Vitis vinifera*. This suggests that PM infection can also stimulate natural defence mechanism in *Vitis vinifera*. The biosynthesis and biological function of rotundone has not been determined, but as with several other sesquiterpenes, it could be involved in the natural defence mechanisms of the vine, especially through the mevalonate/jasmonic acid pathway (D'Onofrio *et al.*, 2009; Dicke and Baldwin, 2010). In the latter study, methyl jasmonate and jasmonic acid induced a wide range of sesquiterpenes in berry cell suspension cultures of Cabernet Sauvignon and Riesling. On the experimental site, a correlation (r^2 = 0.58) was observed between the rotundone concentration in experimental wine and PM severity (Figure 2). As experimental wines were made from healthy berries, these results suggest that infected berries could induce plant defence mechanisms leading to synthesis of rotundone in the neighboring healthy berries. As the correlation observed is positive – with clones 555 showing the lowest PM severity and rotundone in wines – mechanisms involved in the clonal susceptibility discussed previously are more likely to be related to constitutive than to induced factors. Even though in 2013 no pathogen infection (i.e. *Botrytis cinerea*) could be visually detected or identified through determination of gluconic acid (results not shown), our results, which did not show any vintage × clone interaction for rotundone, suggest that clonal differences in susceptibility to biotic stress such as PM through constitutive mechanisms might help explain the differences observed in rotundone between the clones.

In 1998, a Duras N repository containing 150 clones was established in Gaillac. After initial investigations, seven clones were selected to be compared with clone 554 and a new plot dedicated to clonal selection was planted in 2007. Rotundone determinations in wines will be carried out and will bring additional information to most commonly agronomical and enological data for clone selection. If higher concentrations in rotundone were to become a criterion for a new clone selection, then it is important to confirm whether this ability to produce high levels of peppery aroma is related to susceptibility to PM or to any other disease.

Table 3 - Effect of clone on TC, $\delta^{13}C$, classical laboratory analysis of the grape at harvest and production aptitude.

Parameter	Clone number				*Significance*
	554	555	627	654	*P*
Trunk circumference (cm)	19.2 ± 1.2 a	19.6 ± 1.4 a	19.2 ± 1.2 a	19.6 ± 1.8 a	0.574
2013					
200 berry weight (g)	387 ± 9 a	398 ± 8 a	385 ± 5 a	390 ± 8 a	0.260
Sugar concentration (°Brix)	21.1 ± 0.2 a	19.9 ± 0.2 c	20.1 ± 0.3 b	21.0 ± 0.2 a	<0.0001
Total acidity (mEq/L)	163 ± 2 b	175 ± 4 a	157 ± 2 c	158 ± 0 c	0.001
pH	3.02 ± 0.03 a	2.99 ± 0.01 a	3.01 ± 0.02 a	3.03 ± 0.01 a	0.104
Tartaric acid (g/L)	4.21 ± 0.06 a	3.69 ± 0.18 c	3.84 ± 0.06 bc	3.97 ± 0.23 b	0.005
Malic acid (g/L)	7.49 ± 0.16 b	8.42 ± 0.11 a	7.32 ± 0.10 b	7.41 ± 0.11 b	<0.0001
Amino acids (mg/L)	77.2 ± 2.1 a	78.5 ± 0.4 a	75.5 ± 3.7 a	76.1 ± 3.2 a	0.548
$NH4^+$ (mg/L)	38.9 ± 3.9 a	38.2 ± 3.2 a	31.9 ± 3.1 a	39.8 ± 5.6 a	0.150
K^+ (g/L)	1.15 ± 0.08 a	1.16 ± 0.05 a	1.16 ± 0.02 a	1.22 ± 0.06 a	0.438
Total Phenolic Index (TPI)	89.2 ± 2.5 a	73.1 ± 0.4 c	77.7 ± 1.8 b	77.9 ± 1.8 b	<0.0001
Anthocyanin (mg/kg)	1128 ± 48 a	908 ± 58 b	939 ± 24 b	952 ± 40 b	0.001
Number of clusters per vine	16.4 ± 4.1 a	15.6 ± 4.1 a	16.2 ± 5.7 a	16.3 ± 4.7 a	0.939
Yield (kg/vine)	2.29 ± 0.66 a	1.98 ± 0.64 a	2.47 ± 1.01 a	2.20 ± 0.81 a	0.196
$\delta^{13}C$	-28.6 ± 0.0 a	-28.4 ± 0.1 a	-28.5 ± 0.1 a	-28.5 ± 0.1 a	0.287
2014					
200 berry weight (g)	469 ± 22 b	471 ± 17 b	496 ± 20 a	465 ± 2 b	0.002
Sugar concentration (°Brix)	20.7 ± 0.2 a	20.6 ± 0.1 a	20.6 ± 0.2 a	20.6 ± 0.2 a	0.887
Total acidity (mEq/L)	165 ± 6 a	159 ± 5 a	162 ± 3 a	157 ± 3 a	0.193
pH	2.82 ± 0.04 b	2.88 ± 0.04 a	2.83 ± 0.03 b	2.83 ± 0.01 b	0.025
Tartaric acid (g/L)	3.19 ± 0.06 ab	3.34 ± 0.09 a	2.96 ± 0.01 b	3.29 ± 0.23 ab	0.029
Malic acid (g/L)	8.66 ± 0.38 a	8.40 ± 0.34 a	8.63 ± 0.26 a	8.36 ± 0.21 a	0.543
Amino acids (mg/L)	79.4 ± 11.9 a	81.9 ± 8.8 a	95.1 ± 7.7 a	99.2 ± 14.3 a	0.086
$NH4^+$ (mg/L)	35.0 ± 5.8 a	41.5 ± 11.2 a	44.8 ± 9.8 a	42.9 ± 7.9 a	0.383
K^+ (g/L)	1.35 ± 0.05 a	1.30 ± 0.05 a	1.30 ± 0.03 a	1.29 ± 0.01 a	0.235
Total Phenolic Index (TPI)	75.1 ± 6.2 a	74.0 ± 1.4 a	72.4 ± 0.9 a	77.6 ± 1.5 a	0.341
Anthocyanin (mg/kg)	897 ± 62 a	920 ± 69 a	882 ± 5 a	932 ± 8 a	0.365
Number of clusters per vine	11.6 ± 6.1 a	9.1 ± 4.7 a	10.3 ± 4.2 a	11.7 ± 5.1 a	0.186
Yield (kg/vine)	1.46 ± 0.97 a	1.00 ± 0.58 a	1.20 ± 0.64 a	1.13 ± 0.50 a	0.260
$\delta^{13}C$	-28.6 ± 0.2 a	-28.5 ± 0.2 a	-28.4 ± 0.2 a	-28.5 ± 0.0 a	0.929
Severity of powdery mildew (%)	20.0 ± 22.2 c	12.1 ± 15.8 d	33.4 ± 30.7 b	41.5 ± 28.7 a	<0.0001

Means followed by the same letter within a row are not significantly different (Fisher's least significant difference, $P < 0.05$).

CONCLUSION

Our study allowed characterizing the impact of the four certified Duras N clones on grape quality and rotundone in wines. While vintage × clone interactions were observed for most of the classical analytical variables, rotundone concentrations were higher in 2013 and 2014 in clones 554 and 654 in comparison to clone 555, with clone 627 showing intermediate level. Currently, clones 554 and 555 are the most available clones in the nurseries. Thus, winegrowers who are willing to promote rotundone concentration in their red wines from Duras N should consider planting clone 554. In 2014, differences in PM severity were reported between the four clones and PM infection seems to be a key variable to explain the extremely high rotundone concentrations found in wines from the 2014 vintage. In 2014, a positive correlation ($r^2 = 0.58$) was reported on the experimental site between rotundone in wines and PM severity on grapes, which suggests that grapevine defence response to PM could enhance rotundone production in berries. If the differences in Duras N clone susceptibility to PM were to be confirmed, then our results suggest that the mechanisms involved are more likely to be related to constitutive factors rather than induced factors. Our findings also suggest that clonal differences in susceptibility to biotic stress such as PM might help explain the differences observed in rotundone between the clones.

Table 4 - Characterization of the 2013 and 2014 vintages thanks to several climatic indexes and cumulative rainfall calculated over the whole calendar year, the budburst-veraison and veraison-harvest periods.

	Huglin index (IH)	Cool night index (FNv-r)	Mean air temperature (Tv-r)	Maximal air temperature (Txv-r)	Thermal amplitude (Av-r)	Cumulative rainfall (mm)		
						01/01 - 31/12	Budburst - veraison	Veraison - harvest
2013	1901	12.3	17.8	24.7	12.4	700	313	43
2014	2019	13.3	19.2	26.1	12.8	782	347	48

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A 2-year multisite study of viticultural and environmental factors affecting rotundone concentration in Duras red wine

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Résumé : Ces travaux menés sur deux millésimes ont permis de déterminer et de hiérarchiser les facteurs environnementaux et viticoles clés permettant d'expliquer les différences de concentration en rotundone sur 10 vignobles du Gaillacois. En utilisant des modèles de régression par les moindres carrés, nous avons montré que les meilleurs prédicteurs de la concentration en rotundone étaient l'acide gluconique, le cumul de précipitation, l'indice d'Huglin, les heures d'ensoleillement et l'irradiation moyenne au cours de la maturation. Ces résultats sont susceptibles d'aider les structures de production, notamment les caves coopératives, dans leur sélection parcellaire visant à produire des vins poivrés.

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A 2-year multisite study of viticultural and environmental factors affecting rotundone concentration in Duras red wine

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ABSTRACT

Aim: A study was carried out in 2013 and 2014 to determine the key environmental and viticultural variables affecting the concentration of rotundone, the black pepper aroma compound, in *Vitis vinifera* L. cv. Duras red wines at 10 different vineyard blocks.

Methods and results: For each block, data for fruit quality attributes, as well as climatic and agronomical variables, were collected. Rotundone was quantified in wines prepared by microvinification techniques (in a 1-L Erlenmeyer flask). Rotundone concentration varied across blocks from 63 ng/L to 239 ng/L in 2013 and from 25 ng/L to 115 ng/L in 2014. Three separate partial least squares regression models were constructed to predict rotundone concentration in wines in 2013, in 2014, and in both vintages. Gluconic acid, a secondary metabolite of *Botrytis cinerea*, had a substantial contribution to the 2013 and multivintage models, with a negative regression coefficient with rotundone concentration. Other predictors were associated with abiotic factors such as cumulative rainfall, thermal index, hours of sunshine and mean daily irradiation.

Conclusions: Our results indicate that mesoscale climatic variables are the key factors determining rotundone concentration, and also suggest that *Botrytis cinerea* may be involved in rotundone degradation.

Significance and impact of the study: Our findings may assist grape growers producing Duras red wines to select specific vineyard blocks with the aim of producing wines with a desired rotundone concentration. They also open up new fields of investigation into mechanisms involved in possible rotundone degradation by *Botrytis cinerea*.

KEYWORDS

abiotic factors, *Botrytis cinerea*, mesoscale climate, partial least squares regression model, rotundone

Additional tables can be downloaded from https://oeno-one.eu/article/view/2341

INTRODUCTION

Wine is a complex matrix that contains more than 800 aroma compounds (Robinson *et al.*, 2014). Most of the contributors to the varietal aroma of white wines, such as the monoterpenols responsible for floral notes in Muscat varieties (Williams *et al.*, 1981) and the varietal thiols imparting grapefruit and tropical fruit notes in Sauvignon blanc wines (Tominaga *et al.*, 1998), have been widely studied. Knowledge of odorants accounting for the varietal character of red wines, especially free aroma compounds directly extracted from grapes without being released from a precursor, had been limited to green and undesirable aroma methoxypyrazine compounds (Sala *et al.*, 2000) until the discovery of the sesquiterpene rotundone, which is responsible for the black pepper aroma (Wood *et al.*, 2008). Specific anosmia to this potent odorant, which has a detection threshold of 16 ng/L in red wine, has been reported (Wood *et al.*, 2008). In most cases, rotundone is perceived by consumers either positively or in a neutral manner (Geffroy *et al.*, 2018a), and those who appreciate peppery wines are generally wine connoisseurs who pay more than the average consumer for a bottle of wine (Geffroy *et al.*, 2016a).

Aside from Syrah/Shiraz wines, rotundone has so far been identified at very high concentrations (above 100 ng/L) in red wines made from Schioppettino (Caputi *et al.*, 2011), Duras, Pineau d'Aunis (Geffroy *et al.*, 2014), Gamay (Geffroy *et al.*, 2016a) and Maturana tinta (Cullere *et al.*, 2016). Differences in rotundone concentration have been identified between certified clones of Duras, and the grapevine defense response to powdery mildew might result in enhanced rotundone production (Geffroy *et al.*, 2015a). Zhang *et al.* (2016a) have shown that rotundone might not only be produced as a response to herbivore attacks and proposed the existence of other biosynthetic pathways. Rotundone does not appear to be translocated from vegetative tissues (Geffroy *et al.*, 2016b; Zhang *et al.*, 2016a), and in accordance with the hypothesis of local synthesis in grape berries, crop load reduction through grape thinning has no effect on rotundone concentration in wine (Geffroy *et al.*, 2014). The compound is produced by simple aerial or enzymatic oxidation of α-guaiene (Huang *et al.*, 2014; Takase *et al.*, 2016). Although rotundone has been identified at early stages of development in vegetative tissues (Zhang *et al.*, 2016a), it is known to accumulate late in the season in berries, with the concentration increasing from veraison to harvest (Caputi *et al.*, 2011; Geffroy *et al.*, 2014).

Cool and wet vintages promote the production of peppery red wines (Caputi *et al.*, 2011). Soil characteristics (Scarlett *et al.*, 2014), bunch zone air and bunch surface temperatures (Zhang *et al.*, 2015a), vineyard water balance (Zhang *et al.*, 2015b), and vine water status over the veraison-to-harvest period (Geffroy *et al.*, 2014; Geffroy *et al.*, 2015b) have been identified as key variables that explain differences in rotundone concentration between vintages and within a single vineyard block. Thus, the concentration of rotundone has been reported to be enhanced by the water supply through irrigation (Geffroy *et al.*, 2014; Geffroy *et al.*, 2016b). Conflicting results have been reported regarding the effect of bunch exposure, because leaf removal can reduce (Geffroy *et al.*, 2014), increase, or have no effect on rotundone accumulation (Homich *et al.*, 2017; Geffroy *et al.*, 2018b).

The hierarchy between viticultural and environmental factors driving rotundone production, especially temperature and water status, remains unclear. Although several studies have been conducted on rotundone at an intra-block scale (Scarlett *et al.*, 2014; Geffroy *et al.*, 2015b; Zhang *et al.*, 2015a; Bramley *et al.*, 2017), no data are presently available for studies carried out at a mesoscale. To study these aspects and to identify possible correlations between rotundone and vine and climatic variables, a trial was carried out in 2013 and 2014 on 10 *Vitis vinifera* L. cv. Duras vineyard blocks over the Gaillac protected designation of origin in the south-west of France. For each plot, data for the variables were collected or calculated mostly at veraison or between veraison and harvest, due to the late accumulation of rotundone in berries.

MATERIALS AND METHODS

1. Vineyard blocks and vine panels

The characteristics of the 10 commercial dryland vineyard blocks used for this study are shown in Table 1. The plots were selected to capture a wide range of soil and climatic conditions, with the aim of maximizing the range of rotundone concentrations found in the blocks. The 10 blocks were located within the Gaillac

TABLE 1. Characteristics of the 10 vineyard blocks used in the study

Block no.	Clone no.	Rootstock	Plant density (vines/ha)	Training system	Year of plantation	Row orientation	Elevation (m a.s.l.)	Slope (%)
1	554	Gravesac	4545	Guyot	1999	NW to SE	159	1.6
2	554	101-14 MGt	4132	Guyot	2002	NE to SW	118	0.5
3	555	3309C	4545	Guyot	2005	NW to SE	195	4.1
4	555	140R	4545	Guyot	2004	N to S	226	4.0
5	554	3309C	6172	Cordon	2003	N to S	201	4.9
6	554	3309C	4545	Cordon	2005	NE to SW	280	5.5
7	MS	SO4	4761	Goblet	1999	NW to SE	146	2.7
8	554	3309C	4545	Cordon	2001	NW to SE	129	1.2
9	654	SO4	4000	Cordon	1992	NW to SE	174	1.7
10	555	Fercal	4545	Guyot	1996	N to S	271	2.8

E, east; m a.s.l., meters above sea level; MGt, Millardet et de Grasset; MS, massal selection; N, north; S, south; W, west.

protected designation of origin, which covers a surface area of approximately 1000 km^2.

At each block, one 30-vine panel (comprising 10 consecutive vines from three adjacent rows) was selected for data collection and sampling. Vine management was performed by growers, except for fruit-zone leaf removal, which is a practice that is likely to affect rotundone concentration (Geffroy *et al.*, 2014; Homich *et al.*, 2017; Geffroy *et al.*, 2018b). To avoid bias due to differences in the timing and extent of mechanical leaf removal between blocks when the practice had to be performed by growers, all the leaves were removed manually from the basal node to the first node above the top bunch on the morning-sun side of the canopy at mid-veraison, when rotundone starts to accumulate in berries (Caputi *et al.*, 2011). Row orientation was determined using Google Earth Pro software version 7.3.0.3832 (Google Inc., Mountain View, CA, USA). Elevation and slope data were extracted from the RGE ALTI 5 m device, provided by the French National Institute of Geographic and Forest Information.

2. Phenology and vine measurements

For each block, the percentage of veraison was determined by randomly collecting 100 berries from both sides of the row and also several parts of the bunch, and by counting the number of colored berries every third day from the end of July to the end of August. Linear regression tests were performed using Excel software (Microsoft Corp., Redmond, WA, USA) to calculate the onset and end of veraison, the mid-veraison date, and the duration of veraison.

Exposed leaf area (m^2/m^2 soil), which is the leaf area that can be exposed to direct solar radiation, was estimated at mid-veraison using the protocol proposed by Murisier (1996). Measurements were made for six consecutive vines on each of the three rows constituting the 30-vine panel. The ratio between canopy height and row spacing, which is an indicator of the shade of one vine row provided by an adjacent row (Smart, 1985), was also calculated.

For each sampled vine, the number of bunches per vine was counted and the yield at harvest (kg/vine) was monitored by weighing individual crop loads, using a Precia Molen C20 K balance (Precia SA, Privas, France). Mean bunch weights were also calculated for each block.

Leaf area to crop weight ratio, which is an indicator of vine balance and ability to produce high-quality fruit and wines (Kliewer and Dokoozlian, 2005) was calculated as follows:

leaf area to crop weight ratio = [exposed leaf area (m^2/m^2 soil) ´ 10 000] / [yield at harvest (kg/vine) ´ plant density (vines/ha)]

In 2014 only, powdery mildew (*Erysiphe necator*) infections were detected on bunches from vines of one of the blocks. For this vintage, the severity of the infection was assessed at harvest by visually estimating the percentage of the bunch area colonized by the pathogen on 100 grape bunches (50 on each side of the vine). In 2013 and 2014, the severity of bunch rot (*Botrytis cinerea*) was assessed by determination of the gluconic acid content of the grapes (see the section *Berry sampling and analyses*), which correlates with the visually estimated percentage of rotten grapes (Bocquet et al., 1995).

Pruning wood weight (kg/vine) was estimated by weighing 1-year-old shoots of 10 out of 30 vines in January 2014 and 2015, using a Precia Molen C20 K balance.

3. Weather measurements and water-balance model

Daily high-resolution data provided by Météo France (Toulouse, France), the French weather service, were obtained using ANTILOPE (Champeaux et al., 2009) for rainfall and AROME (Brousseau et al., 2011) for air temperature and evapotranspiration. Resolutions were 1 km^2 and 4 km^2 for ANTILOPE and AROME, respectively. These data were used to calculate the average minimum, maximum and mean daily air temperature for each block between veraison and harvest; to calculate the Huglin index over the period from 1 April to 30 September (Tonietto and Carbonneau, 2002); to determine cumulative rainfall over different periods; and to run a water-balance model.

The percentage of soil covered by grass differed greatly between the blocks, and the water balance for intercropped systems (WaLIS) model was used to simulate the stem water potential (ψ_{stem}) of the vine (Celette et al., 2010; Dufourcq et al., 2013) at different time points between 15 days before mid-veraison and harvest for each block. The minimal and maximal values of ψ_{stem} over the mid-veraison to harvest period were also identified for each block. Calibration of the model was carried out in 2013 and 2014 based on three measurements of ψ_{stem} that were obtained between the beginning of July and the end of August for each block. Each measurement was taken from 3 of the 30 vines and from two mature exposed leaves per vine, according to the protocol proposed by Choné et al. (2001). These three vines were marked, and ψ_{stem} was always measured on these selected plants. On cloudless days, leaf blades were initially enclosed in plastic bags between 11:00 and 12:00 hours, and measurements were made between 14:00 and 15:00 hours.

The hours of sunshine, solar irradiation, and hygrometry data over the mid-veraison to harvest period, with a 9-km^2 spatial resolution, were obtained for each block from HelioClim-3, a satellite-based surface solar irradiation database (Blanc et al., 2011; Eissa et al., 2015).

In April 2015, just before budburst, trunk circumference, which is an index of vine vigor that correlates well with the rotundone concentration in vines within the same block (Geffroy et al., 2015b), was determined for every vine within each block. We used the average of measurements obtained at three different heights: just above the graft, 10 cm under the trellis wire, and halfway between the two first points of measurement.

4. Berry sampling and analyses

Previous modeling of the kinetics of rotundone accumulation in berries has shown that the concentration increases from veraison and reaches a plateau after a certain time (Zhang et al., 2015b). According to Zhang et al., this plateau is higher and is reached earlier during cool and wet seasons. In 2011 and 2012, two hot and dry vintages that did not promote high rotundone concentration in the grapes of vines grown in the south-west of France, this plateau was achieved for Duras wines 44 days after mid-veraison (Geffroy et al., 2014).

To collect grapes with the maximum rotundone concentration, each block was harvested in 2013 and 2014 exactly 44 days after mid-veraison. For each block and for each year, two samples, consisting of 200 berries from both sides of the row and several parts of the bunch (four berries per vine on each side of the row), were collected.

Before the start of the analysis, the mass of the 200 berries in each sample was measured. The berries were then crushed gently, and the musts obtained were centrifuged (14,000 g for 6 min) to enable conventional laboratory and $\delta^{13}C$ analyses. Sugar concentration (°Brix) was determined with a PAL digital handheld pocket refractometer (Atago, Tokyo, Japan). Titratable acidity was measured according to the methods of the Organisation Internationale de la Vigne et du Vin (2009), and pH was measured with an HI 3221 pH meter (Hanna Instruments, Woonsocket, RI, USA).

A Konelab Arena 20 sequential analyzer (Thermo Fisher Scientific, Waltham, MA, USA), in conjunction with enzyme kits provided by several suppliers, was used to determine the concentration of amino acids, ammonium, gluconic acid (Megazyme, Bray, Ireland) and malic acid (Thermo Fisher Scientific). Potassium concentration was determined by flame photometry (Bio Arrow, France) according to the

TABLE 2. Variables for which data were collected

Block	Phenology	Vine	
Variable	Variable	Variable	Period of measurement
Age of the plot	15 days before veraison	Pruning wood weight	Winter
Slope	Onset of veraison	Trunk circumference	Winter
Clone	Mid-veraison	Exposed leaf area	Veraison
Rootstock	End of veraison	Height-to-row spacing ratio	Veraison
Training system	Duration of veraison	Leaf area to crop weight ratio	Veraison/harvest
Row orientation		Stem water potential (ψ_{stem})	15 days before veraison
Plant density		Stem water potential (ψ_{stem})	Onset of veraison
Elevation		Stem water potential (ψ_{stem})	Mid-veraison
		Stem water potential (ψ_{stem})	End of veraison
		Stem water potential (ψ_{stem})	Harvest
		Stem water potential (ψ_{stem})	Minimum veraison – harvest
		Stem water potential (ψ_{stem})	Maximum veraison – harvest
Crop		**Climate**	
Variable	Period of measurement	Variable	Period of measurement
Yield per vine	Harvest	Mean air temperature	Veraison – harvest
No. of bunches per vine	Harvest	Minimum air temperature	Veraison – harvest
Bunch weight	Harvest	Maximum air temperature	Veraison – harvest
Sugar concentration	Veraison,[a] harvest	Huglin index	1 April – 30 September
Mass of 200 berries	Harvest	Mean air hygrometry	Veraison – harvest
Titratable acidity	Veraison,[a] harvest	Mean daily irradiation	Veraison – harvest
pH	Veraison,[a] harvest	Hours of sunshine	Veraison – harvest
Malic acid concentration	Veraison,[a] harvest	Cumulative rainfall	1 January – 30 December
Tartaric acid concentration	Harvest	Cumulative rainfall	1 April – 30 September
Amino acids concentration	Veraison,[a] harvest	Cumulative rainfall	15 days before veraison – veraison
Ammonium concentration	Veraison,[a] harvest	Cumulative rainfall	Veraison – harvest
Total phenolic index	Harvest	Evapotranspiration	Veraison – harvest
Anthocyanins content	Harvest		
$\delta^{13}C$	Veraison,[a] harvest		
Gluconic acid concentration	Harvest		
Severity of powdery mildew	Harvest[a]		
Skin-to-juice ratio	Harvest		

[a]Determined only in 2014 and excluded from the multivintage model. Data included in the partial least squares regression models to predict rotundone concentration in wine

methods of the Organisation Internationale de la Vigne et du Vin (2009), and the concentration of tartaric acid was determined by colorimetric titration (Hill and Caputi, 1970). $\delta^{13}C$, a proxy for vine water status (van Leeuwen *et al.*, 2009) was determined at harvest according to the protocol described by Geffroy *et al.* (2014). All the analytical determinations were carried out twice.

The berry skins that had not been used for the previous analyses were crushed for 2 min at low speed (600 rpm) using a Faciclic food blender (Moulinex, Ecully, France), in preparation for phenolic analyses. The concentration of anthocyanins and the total phenolic index of the grapes were quantified according to the method described by Cayla *et al.* (2002). In 2014 only, samples of 240 berries were also collected from the 10 blocks at mid-veraison to determine the weight of 200 berries, $\delta^{13}C$, sugar concentration, titratable acidity, pH, concentration of malic acid and amino acids, and ammonium content, according to previously described methods.

5. Microvinification and rotundone analysis

Rotundone content was analyzed indirectly in wines fermented under microvinification conditions. For each block and for each year, two grape samples, comprising 800 g of berries from both sides of the row and several parts of the bunch, were collected at harvest 44 days after mid-veraison. Microscale fermentation was carried out in a 1-L Erlenmeyer flask, according to the protocol described by Geffroy *et al.* (2014). After 8 days of fermentation, the amount of wine and of skins was measured to determine the skin-to-juice ratio at pressing (%). The wines were centrifuged (14,000 *g* for 6 min), and sulfite (80 mg/L) was added before bottling and storage at 4°C or less until the rotundone analysis.

The rotundone concentration of the wines was determined by solid-phase microextraction-multidimensional gas chromatography–mass spectrometry, according to the protocol described by Geffroy *et al.* (2014). The analyses were performed in two different years but during the same period of each year, to reduce potential variation associated with different post-bottling times.

6. Data treatment

Details of the variables for which data were collected are shown in Table 2. Rotundone concentration and data for the other variables, collected for the 10 blocks over the 2 years of the study, were subjected to analysis of variance, with the vintage as a factor and blocks as repetitions, using Xlstat software version 19.5 47062 (Addinsoft, Paris, France). Fisher's least significant difference test was used as a post-hoc comparison of means at *P* £ 0.05.

Based on previous work conducted with the aim of modeling 3-isobutyl-2-methoxypyrazine concentration in Cabernet Franc grapes (Scheiner *et al.*, 2011), a multivariate analysis using partial least squares regression (PLSR) was carried out to identify key factors affecting rotundone concentration in wine. The data were averaged over the replicates before PLSR was performed. For the multi-vintage model, variables that had been determined only in 2014 were discarded. A first set of PLSR for which the number of latent variables was determined by the lowest predicted residual sum of squares was conducted, using Xlstat software to model rotundone concentration in 2013, in 2014, and in both vintages (Wold *et al.*, 1984). Variables with a variable importance in projection score below 1 were removed manually and the model was regenerated. Once the number of variables remaining in the model was lower than the number of observations (10 for 2013 and 2014, and 20 for the multi-vintage data set), PLSR was conducted with Unscrambler X software version 10.4 (Camo, Oslo, Norway).

The results were validated by a full cross-validation (leave-one-out) procedure. Variables that did not contribute sufficiently to the model (absolute values of the loading weights below 0.1) were removed manually and the model was regenerated. For the multivintage model, one outlier (block no. 6 in 2013) detected using principal component analysis was removed.

RESULTS AND DISCUSSION

The data collected for each vineyard block in 2013 and 2014 are presented as supplementary material (Supplementary tables 1 and 2).

1. Main differences between the vintages

The 2013 vintage was characterized by a cold spring and regular and heavy rainfall events over the vine vegetative cycle that resulted in severe bunch rot (*Botrytis cinerea*) damage, ripening difficulties and delay of harvest. The mean harvest date for the 10 blocks was 6 October ± 4 days. In 2014, the winter was rainy and warm and summer was extremely rainy between mid-July and the end of August, whereas conditions during ripening were dry and hot. The mean harvest date was 22 September ± 3 days. Compared with 2014, the 2013 vintage was significantly wetter as well as cooler over the entire winegrowing and ripening periods, as reflected by the cumulative rainfall between 1 April and 30 September (423 ± 22 mm in 2013, 358 ± 19 mm in 2014) and between veraison and harvest (115 ± 18 mm in 2013, 63 ± 13 mm in 2014), the Huglin index (1862 ± 51 in 2013, 1969 ± 48 in 2014), and the mean air temperature over the veraison-to-harvest period (18.50 ± 0.56°C in 2013, 19.48 ± 0.42°C in 2014). The values of ψ_{stem} were significantly lower at mid-veraison during the first year of the study (–0.87 ± 0.20 MPa in 2013, –0.60 ± 0.12 MPa in 2014). Owing to the larger quantities of rainfall received in 2013 over the veraison-to-harvest period, the level of water deficit was higher at harvest in 2014

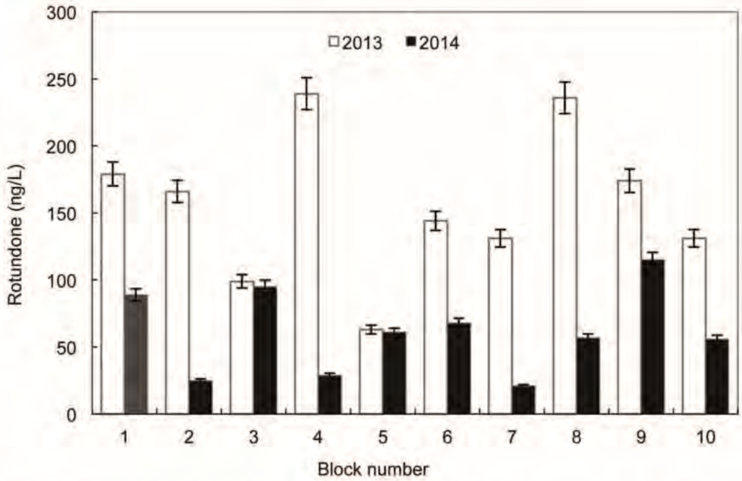

FIGURE 1. Rotundone concentration in wines made from grapes from the 10 vineyard blocks of Duras N in 2013 and 2014.

(–0.51 ± 0.17 MPa in 2013, –1.06 ± 0.14 MPa in 2014).

Vintage had a weak effect on crop and grape analytical characteristics, because no significant differences in yield (1.75 ± 1.01 kg/vine in 2013, 2.65 ± 1.22 kg/vine in 2014), sugar concentration (21.8 ± 0.9°Brix in 2013, 21.8 ± 0.6°Brix in 2014), pH (3.06 ± 0.12 in 2013, 3.05 ± 0.07 in 2014), titratable acidity (9.20 ± 1.13 g/L as tartaric acid in 2013, 9.10 ± 1.22 g/L as tartaric acid in 2014), amino acid concentration (120 ± 33 mg/L in 2013, 136 ± 48 mg/L in 2014) or total phenolic index (79.6 ± 10.8 in 2013, 88.5 ± 15.2 in 2014) were observed between 2013 and 2014. Concentrations of anthocyanins (872 ± 143 mg/L in 2013, 1048 ± 194 mg/L in 2014) and ammonium (40.9 ± 20.3 mg/L in 2013, 63.8 ± 20.4 mg/L in 2014) were the only fruit compound variables that differed significantly between the two vintages.

Rotundone concentration was significantly higher ($P < 0.001$) in the cooler and wetter 2013 growing season (Figure 1). This result is consistent with those of previous studies (Caputi *et al.*, 2011). Surprisingly, some blocks that had high rotundone concentration in 2013 (i.e. blocks no. 2 and no. 8), with values among the highest ever reported for Duras, had low to moderate concentrations in 2014. In the same way, some blocks that had high concentrations in 2014 (i.e. blocks no. 3 and no. 5) had low to moderate rotundone concentrations in 2013. This observation runs counter to the belief that some vineyards within a given wine region may always be prone to yielding wines with substantial amounts of rotundone. This led us to the notion that qualitative or quantitative fixed variables such as year of plantation, elevation, training system, clone or rootstock do not make a substantial contribution to the rotundone model.

2. Partial least squares regression rotundone models

Basic statistics of the models, regression coefficients, means and standard deviations of the variables included in the best models, are shown in Table 3. Regression plots of predicted rotundone concentration versus observed rotundone concentration in 2013, in 2014, and in both vintages are presented in Figure 2. The performance of the 2013 vintage model, reflected by its values of R^2 and root mean square error, was better than that of the other models. However, all the models appear to be appropriate for predicting rotundone concentration using a limited number of input variables for blocks with low or high rotundone concentrations.

The loadings of the latent variables used in the regression models are shown in Table 4. The first latent variable in the 2013 model was mainly characterized by gluconic acid concentration. For 2013, this variable was

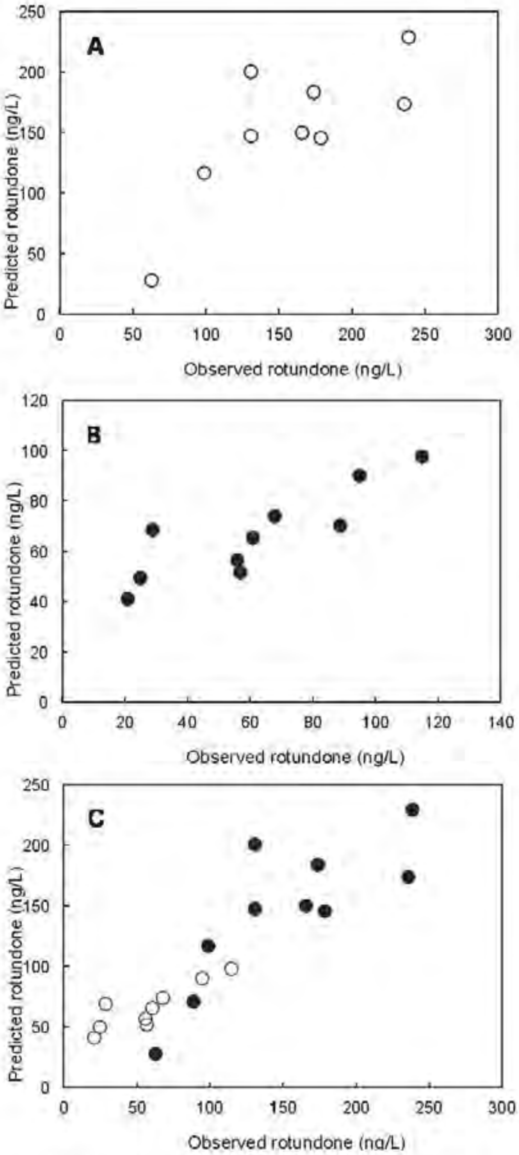

FIGURE 2. Predicted versus observed rotundone concentration.

Predicted versus observed rotundone concentration, using data from the models described in Table 3: A, 2013 ($R^2 = 0.95$); B, 2014 ($R^2 = 0.78$); and C, multi-vintage ($R^2 = 0.81$).

significantly negatively correlated with rotundone concentration ($P < 0.01$, $R^2 = 0.69$) (Figure 3). It also contributed to the multi-vintage model. This observation was somewhat unexpected, because laccase, the polyphenol oxidase produced by *Botrytis cinerea*, has the ability to convert α-guaiene to rotundone via a non-specific oxidation reaction in the presence of chemical mediators (Schilling *et al.*, 2013). However, it has also been documented that *Botrytis cinerea* can withstand the toxic effects of plant compounds such as sesquiterpenes by laccase-mediated oxidation and detoxification (Mayer and Staples, 2002). Further studies will be necessary to confirm this hypothesis and to better understand the mechanisms involved in rotundone degradation by the fungus.

Other variables contributing to the latent variables of the models were associated with abiotic factors, which indicates that mesoscale climate is the key factor driving rotundone production. Notably, the Huglin index, a thermal index based on a degree-day approach from 1 April to 30 September, was a strong contributor to the 2014 model. This is consistent with the finding that cool vintages promote rotundone accumulation in grapevine berries (Caputi *et al.*, 2011). A contribution to the models from mean air temperature between veraison and harvest would have been expected, because post-veraison berry surface temperature has been identified as the main determinant of rotundone concentration in grape berries (Zhang *et al.*, 2015a).

Bunch temperature does not fully reflect air temperature, because it is also determined by absorbed radiation and convective heat loss (Dry, 2009). The extent to which bunch temperature exceeds air temperature depends on degree of exposure, radiation load, wind velocity, berry or bunch size, berry color and bunch compactness. With the exception of wind velocity, which was not assessed, most of the data for these variables were collected directly or depend on data collected for other variables, such as bunch weight, mass of 200 berries, training system, rootstock, row orientation and exposed leaf area. If bunch temperature were the key variable driving rotundone concentration, the best rotundone model would include air temperature and other variables affecting bunch exposure and penetration of solar radiation through the canopy. It has recently been reported that the percentage of degree hours above 25°C (Dh_{25}) in the fruit zone from veraison to harvest correlates with rotundone concentration in berries (Zhang *et al.*, 2015a). Our study was designed and conducted 2 years before publication of these findings. Dh_{25} could not be calculated *a posteriori*, because collection of the data for this variable would

TABLE 3. Basic statistics of the partial least squares regression models

Variable	Model			Mean and standard deviation of model	
	2013	2014	2013–2014	2013	2014
Cumulative rainfall, veraison – harvest (mm)	−0.90[a]	NI	NI	115 ± 18*	63 ± 13
Hours of sunshine	0.72[a]	NI	0.61[a]	273 ± 17	362 ± 5*
Gluconic acid concentration (mg/L)	−0.83[a]	NI	−0.001[a]	72.4 ± 69.1*	14.3 ± 21.2
Cumulative rainfall, 1 April – 30 September (mm)	NI	0.85[a]	0.77[a]	423 ± 22*	358 ± 19
Huglin index	NI	−0.63[a]	NI	1862 ± 51	1969 ± 48*
Cumulative rainfall, 1 January – 31 December (mm)	NI	NI	0.66[a]	19.1 ± 12.6	50.1 ± 7.9*
Mean daily irradiation, veraison – harvest (W/m^2)	NI	NI	0.59[a]	1505 ± 81	1863 ± 50*
No. of latent variables	2	2	2	NA	NA
Root mean square error	11.4	14.1	28.5	NA	NA
R^2 (calibration)	0.95	0.78	0.81	NA	NA
Root mean square error of cross-validation	14.9	20.8	41.3	NA	NA
R^2 (validation)	0.92	0.53	0.59	NA	NA

NA, not applicable; NI, variable not included in the model. *Significantly higher of the means for 2013 and 2014 (least significant difference, P £ 0.05). [a]Regression coefficient between the variable and rotundone concentration
Regression coefficients, means and standard deviations of variables for the best rotundone models

TABLE 4. Loadings of the variables contributing to the best rotundone models

Variable	2013		2014		2013–2014	
	LV 1	LV 2	LV 1	LV 2	LV 1	LV 2
Gluconic acid concentration	−0.99	0.32	NI	NI	0.17	−0.84
Hours of sunshine, veraison – harvest	0.11	0.78	NI	NI	−0.21	0.05
Cumulative rainfall, veraison – harvest	−0.18	−0.55	NI	NI	NI	NI
Cumulative rainfall, 1 April – 30 September	NI	NI	0.26	0.88	0.14	0.20
Huglin index	NI	NI	−1.00	0.48	NI	NI
Cumulative rainfall, 1 January – 31 December	NI	NI	NI	NI	0.43	0.42
Mean daily irradiation, veraison – harvest	NI	NI	NI	NI	0.88	0.29
Explained variance (%)	76.1	19.1	51.9	25.7	40.8	39.9

LV, latent variable; NI, variable not included in the model.

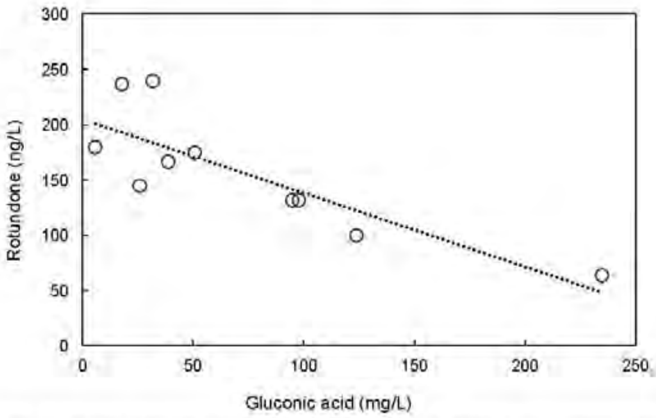

FIGURE 3. Relation between gluconic acid concentration and rotundone concentration.
Relation between gluconic acid content and rotundone concentration in wines in 2013 (n = 10). Linear regression model: y = 0.6667x + 204.47, P < 0.01, R^2 = 0.69.

have required the use of temperature loggers located within the canopy. We cannot completely exclude the possibility that Dh_{25} would have been included in the models.

Mean daily irradiation between veraison and harvest had a substantial contribution to the multi-vintage model. Hours of sunshine was also included in the best rotundone models for 2013 and 2013–2014. These two variables, reflecting the quantity of ultraviolet radiation received by the blocks, were positively correlated with rotundone concentration. Several studies have investigated the light dependency of sesquiterpene emission, with evidence that emission of some sesquiterpenes is solely temperature-controlled whereas emission of others is also affected by light (Duhl *et al.*, 2008). Although additional studies would be needed to confirm this hypothesis, our data suggest that ultraviolet rays may stimulate the production of rotundone. This conclusion is consistent with recent research conducted in cool-climate vineyards on Noiret and Fer cultivars, indicating that grape exposure by leaf removal could promote rotundone accumulation (Homich *et al.*, 2017; Geffroy *et al.*, 2018b).

The amount of rainfall between 1 January and 31 December and during the period of vine vegetative growth (between 1 April and 30 September) had a positive contribution to the 2014 and multi-vintage models, whereas rainfall during the late stage of development (i.e. between veraison and harvest) was negatively correlated with rotundone concentration in 2013. These results are somewhat unexpected, because irrigation around veraison, when rotundone starts to accumulate in berries, stimulates its production (Geffroy *et al.*, 2014; Geffroy *et al.*, 2016b). For the 2013 rainy vintage, even if skin-to-juice ratio and mass of 200 berries were not included in the model, we cannot exclude the possibility that the large excess of water may have resulted in a dilution effect through an increase in berry size.

The contribution to the models from cumulative rainfall between 1 April and 30 September and between 1 January and 31 December deserves further comment. Rotundone is found at elevated concentrations in the early berry stages, notably in flower caps (Zhang *et al.*, 2016b). Rotundone concentration then decreases to reach a minimal concentration at veraison, before increasing again from veraison to harvest (Zhang *et al.*, 2016b). It could be assumed that the precipitation regimen over the entire berry development period, not only during the last stage of maturation, may have affected the final concentration of rotundone through degradation or emission before veraison. Another hypothesis is that increased water availability over the period of vegetative growth and between 1 January and 31 December would have led to higher-vigor vines, resulting in greater bunch shading and lower bunch-zone air temperature. However, this hypothesis is unlikely, because the level of water deficit experienced by the vines at mid-veraison, when shoot growth stops, was minor ($\psi_{stem} = -0.87 \pm 0.20$ MPa in 2013, $\psi_{stem} = -0.60 \pm 0.12$ MPa in 2014) and insufficient to affect vine vegetative expression (van Leeuwen *et al.*, 2009). Additionally, no significant correlation at $P < 0.05$ could be established for the whole data set between cumulative rainfall and either pruning wood weight or exposed leaf area.

Consistent with the fact that the hierarchy of blocks for rotundone concentration can differ greatly from one vintage to the other, none of the rotundone models included any fixed variables. This is somewhat unexpected for the clone, because higher rotundone concentrations have been reported in Duras wines from clones 554 and 654 than from clone 555 (Geffroy *et al.*, 2015a). In the same way, the rootstock could have affected the concentration of rotundone in wine. The rootstock is likely to affect some key variables driving rotundone production, such as scion water status and bunch surface, through its effect on vine vigor, leaf area and penetration of solar radiation (Koundouras *et al.*, 2008). However, it may be difficult to draw firm conclusions, due to the small number of observations for each level of these two factors and the fact that not all clone–rootstock combinations were available.

It has been suggested that the grapevine defense response to powdery mildew could enhance rotundone production in berries (Geffroy *et al.*, 2015a). This hypothesis could not be confirmed under in the present study, because severity of powdery mildew infection was not included in the 2014 model. Period of infection by *Erysiphe necator* was not assessed in that previous study nor in the present study. On the one hand, as rotundone accumulates around veraison, differences in timing of infection, which usually occurs when bunches are receptive (from blooming to veraison), may play a role in defense mechanisms leading to production of rotundone. On the other hand, powdery mildew damage was noticed only in 2014 at just one block, and it remains difficult to draw firm conclusions regarding the contribution of this biotic factor.

Consistent with the findings that rotundone is not translocated from vegetative tissues (Geffroy *et al.*, 2016b; Zhang *et al.*, 2016a) and that its accumulation does not depend on source–sink relations (Geffroy *et al.*, 2014), it should be pointed out that the leaf area to crop weight ratio was not included in any of the rotundone models.

The absence of contribution to the models from sugar concentration at harvest is somewhat unexpected. Although recent studies have shown that an increased alcohol content of wine improves rotundone extraction rate from grapes into wine (Zhang *et al.*, 2017), our data suggest that ethanol concentration in the resulting wines had no effect on rotundone concentration. This may be explained by the relatively small amplitude of difference between the lowest and highest sugar concentrations (20.1°Brix and 22.8°Brix, respectively) recorded between blocks for the 2 years of the study.

We cannot rule out the possibility that some blocks were harvested in 2013 and 2014 before rotundone concentration reached its maximum. This would mean that the proposed models would be valid for prediction of rotundone concentration only in wines from grapes harvested exactly 44 days after mid-veraison and may not be applicable to grapes harvested beyond that time. However, this hypothesis is unlikely, because 2013 and 2014 were characterized by substantial concentrations of rotundone in Duras wines (Geffroy *et al.*, 2016b). Therefore, considering previous findings (Zhang *et al.*, 2016a), the rotundone plateau and the maximum rotundone concentration were expected to have been reached earlier than in 2011 and 2012, and before 44 days after mid-veraison.

With only 2 years of data, we cannot completely disregard the possibility that our model may not be generalizable. This is particularly true because the two vintages of the study were relatively cool and wet for the area, as illustrated by the data for Huglin index and cumulative rainfall between 1 April and 30 September. The 2015 vintage, which was hotter and dryer, was characterized by a different climatic pattern than that of 2013 and 2014. The equation of the multi-vintage model was used to predict rotundone concentration in wine fermented under the same microvinification conditions from grapes collected in 2015 on a Duras plot 44 days after mid-veraison. Based on measurements made on this block for mean daily irradiation between veraison and harvest (1930 W/m^2), hours of sunshine between veraison and harvest (419), cumulative rainfall between 1 April and 30 September (348 mm), cumulative rainfall between 1 January and 31 December (516 mm) and gluconic acid concentration (12 mg/L), the predicted rotundone concentration was 30.8 ng/L; the observed value was 26.0 ng/L. In light of this prediction, we believe that the model may be sufficiently robust to also allow estimation of rotundone concentration during hot vintages.

CONCLUSION

Our study allowed us to model and assess the effect of environmental and viticultural factors on rotundone concentration in wine. Gluconic acid, a secondary metabolite of *Botrytis cinerea* that is negatively correlated with rotundone concentration, was included in the best rotundone model for the 2013 vintage and multi-vintage (2013–2014) models. This observation suggests that the fungus may be involved in rotundone degradation mechanisms, probably through its laccase activity. Other variables included in the PLSR models were abiotic factors. This indicates that mesoscale climate is the key factor driving rotundone production. In most cases, cumulative rainfall and thermal index for the entire vine vegetative period seem to be better predictors than variables calculated during the maturation period only. The positive contribution of mean daily irradiation between veraison and harvest and hours of sunshine suggest that rotundone production may be stimulated by ultraviolet exposure. Our findings may assist grape growers producing *Vitis vinifera* L. cv. Duras red wines from vineyards scattered across the studied viticultural area, to select specific blocks with the aim of producing wines with a specific rotundone concentration. They also open new fields of investigation into the mechanisms involved in possible rotundone degradation by *Botrytis cinerea*.

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Effect of maturity and viticultural techniques on the rotundone concentration in red wine made from *Vitis vinifera* L. cv. Duras

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Résumé : Ces travaux ont permis d'étudier l'impact de plusieurs dates de récolte et de pratiques viticoles sur la concentration en rotundone de vins de Duras N. Nos résultats mettent en avant que la concentration en rotundone augmente avec la maturation puis atteint un plateau 44 jours après la véraison. Aucun effet d'une pulvérisation d'acide jasmonique ni d'un éclaircissage n'a été mis en évidence. Si un effeuillage pratiqué sur les deux faces du rang à la véraison a fortement pénalisé la concentration en rotundone dans les vins, 4 apports de 12 mm d'eau d'irrigation ont favorisé son accumulation. Par ailleurs, nous avons identifié au niveau intra-parcellaire, une corrélation entre la concentration en rotundone et le $\delta^{13}C$ déterminé sur le sucre des raisins.

Abstract

Background and Aims: Rotundone, a potent aroma compound responsible for peppery aroma and flavor in Shiraz, was detected recently in *Vitis vinifera* L. cv. Duras wines. In 2011 and 2012, two separate experiments were carried out to determine the effect of maturity and viticultural techniques on rotundone concentration in Duras wine.

Methods and Results: Rotundone was measured in wines prepared by micro-vinification techniques (1 L Erlenmeyer). Accumulation of this compound in wines depended on vintage conditions and the degree of ripening, such that a higher concentration of rotundone was reached 44 days after mid-veraison. Application of exogenous jasmonic acid and grape thinning did not significantly affect rotundone concentration, whereas leaf removal strongly reduced rotundone concentration. Wine from the irrigated treatment had a higher concentration of rotundone and in most cases, vine water status over the veraison–harvest period was identified as a key variable that was well correlated with observed intra-plot variability.

Significance of the Study: These findings confirm that some common viticultural practices can be used to manipulate the peppery character of Duras wines. Our results may also assist grape growers to produce Syrah and other cultivars where rotundone makes an important contribution to wine aroma.

Keywords: *maturity, peppery aroma, rotundone, viticultural techniques,* Vitis vinifera *L. cv. Duras*

Introduction

Rotundone is a potent aroma compound which was identified for the first time in 2008 in Shiraz wine (Siebert et al. 2008, Wood et al. 2008). The compound has a low aroma detection threshold of 8 ng/L in water and 16 ng/L in red wine (Siebert at al. 2008, Wood et al. 2008), and it has been described by Ferreira (2012) as one the 16 most important aroma compounds in wine. Specific anosmia to this compound has been reported; for example, approximately 20% of panelists could not detect this compound during the first sensory trials at the highest concentration tested (4000 ng/L), even in water (Wood et al. 2008). Since then, rotundone has been identified in an increasing number of vine cvs, including Grüner Veltliner, Vespolina, Schioppettino (Caputi et al. 2011, Mattivi et al. 2011), and Durif and Graciano (Herderich et al. 2012). A high concentration of rotundone was recently recorded by the Institut Français de la Vigne et du Vin in French grape cvs, such as Pineau d'Aunis (up to 200 ng/L) and Gamay (up to 88 ng/L) grown in cool climate vineyards (our unpublished data). Siebert and Solomon (2011) reported that rotundone was located in Shiraz grape berry exocarp or skin whereas none was detected in the pulp or seeds and this is in agreement with Caputi et al. (2011) when studying Vespolina. Studies have also highlighted the increase in rotundone from full veraison to harvest, with a higher concentration most likely to accumulate in grapes in cooler vintages and in cooler vineyards (Caputi et al. 2011, Logan et al. 2011, Siebert and Solomon 2011). The biosynthesis and biological function of rotundone has not been determined, but as with several other sesquiterpenes it could be involved in the vine's natural defense mechanisms, especially through the mevalonate/jasmonic acid pathway in response to insect attacks (D'Onofrio et al. 2009). In that study, methyl jasmonate and jasmonic acid induced a wide range of sesquiterpenes in berry cell suspension cultures of Cabernet Sauvignon and Riesling.

Plant sesquiterpene biosynthesis is known to be controlled by abiotic factors (Dulh et al. 2007) and like other grape derived aroma compounds, rotundone accumulation in grapes could depend upon environmental and viticultural factors. It has been shown that canopy microclimate and bunch light exposure (Marais et al. 1999), crop load (Chapman et al. 2004) and water and nitrogen status (Peyrot des Gachons et al. 2001, Choné et al. 2006) can modify the composition of berry aroma compounds, such as 3-alkyl-2-methoxypyrazines, monoterpenes, norisoprenoides and varietal thiols.

Vitis vinifera L. cv. Duras is the most planted red grape cultivar in the Protected Designation of Origin (PDO) Gaillac in the south-west of France. 'Black pepper' is a common aroma descriptor used to describe the aroma of Duras wines. A preliminary study was conducted in early 2011 by the Institut Français de la Vigne et du Vin, in collaboration with The Australian Wine Research Institute, to confirm the presence of rotundone in three Duras wines from the 2008 vintage. The concentration of rotundone varied between 25 and 95 ng/L with a good correlation between analytical values and sensory intensity of the peppery character in the wines. Following these results, a study was conducted with this cv. in 2011 and in 2012 in order to: (i) validate the kinetics of accumulation of rotundone during ripening, as observed for other cvs grown under different pedoclimatic conditions; (ii) study the impact of three viticultural practices (leaf removal, grape thinning and irrigation) on the rotundone concentration in wine; and (iii) study the impact of exogenous jasmonic acid application on rotundone biosynthesis.

Material and methods

Experimental design and site

Two separate experiments, the first one concerning maturity and the second one viticultural techniques, were established in two separate parts of the same vineyard. The 0.50 ha vineyard located in the heart of the Gaillac region (lat. 43° 50' 37'' N; long. 01°

51' 20'' E) was selected because a high concentration of rotundone (95 ng/L) was recorded in 2008 in the wine from this site. This vineyard, typical of the area with 2.20 m x 1 m vine spacing, was planted in 1999 with Duras (clone designated number 554) grafted on Gravesac rootstock. Orientation of the vine rows was north-west to south-east and vines were trained with vertical shoot positioning (VSP) on a single Guyot pruning system. The soil was managed by chemical weed control under the vines, and by grass cover or mechanical weeding in every second inter-row. The experiments exploring the impact of maturity and viticultural techniques were undertaken using a randomised complete block design with three replications. For both experiments, the experimental plot consisted of five rows, and each experimental unit of 26.4 m^2 contained of twelve continuous vines. The experimental plot presented with a slope of 5%.

Weather measurements

In 2011 and 2012, rainfall and air temperature (minima, maxima and mean values) were monitored by a CimAGRO weather station (Cimel Electronique, Paris, France) placed within 200 m from the experimental site. In order to characterise the two vintages, several indexes proposed by Tonietto and Carbonneau (2002) were calculated: Huglin index or heliothermal index (IH) over the period of 1 April to 30 September; the cool night (FNv-r); the mean air temperature (Tv-r); the maximal air temperature (Txv-r); and the thermal amplitude (Av-r) indexes over the veraison-harvest period.

Effects of maturity

Wines were made from grape samples picked at five times during ripening, from after mid-veraison. Mid-veraison was determined by collecting randomly 100 berries on the experimental site and by counting the number of colored berries every third day from the end of July to the beginning of August. Mid-veraison occurred in 2011 on 1 August and in 2012 on 4 August. The times chosen after mid-veraison were: 30 days (50%

ver.+30); 37 days (50% ver.+37); 44 days (50% ver.+44); 51 days (50% ver.+51); and 58 days (50% ver.+58). In the south-west of France, commercial harvest of Duras typically commences about 45 days after mid-veraison.

Effects of viticultural treatments.

Four viticultural techniques were investigated and compared to a control treatment.

- 100% fruit zone leaf removal (Leaf) was done by hand on both sides of the row at mid-veraison. All leaves were removed from the basal node to the first node above the top bunch.
- Bunch thinning (Grape) was conducted with an intensity of 40% at mid-veraison using grape snips. For each individual vine, the total number of bunches was counted. A rate of 40% was applied and the number of bunches to be removed was then determined by rounding the result of the calculation to the closest integer number. Bunches to be thinned were selected in order to homogenise maturity and to avoid overcrowding.
- Irrigation (Irrigation) was started about 10 days before expected mid-veraison date. Four irrigations, each equivalent to 10 mm of rain, were applied every week using drip irrigation pipes (6 L/h drippers at 0.5 m spacing). Irrigation volumes were calculated as follows:

$$\text{Irrigation volume}\ (\text{L/vine plant}) = \frac{\text{Equivalent of rainfall}\ (\text{mm}) \times \text{Surface of the plot}\ (\text{m}^2)}{\text{Number of vine plants of the plot}} \quad (1)$$

- The exogenous jasmonic acid (Elicitor) was applied 20 days after mid-veraison, at 200 L/ha with a 1 mmole/L solution prepared with jasmonic acid provided by Sigma-Aldrich (St Louis, MO, USA) and supplemented with Tween 20 at 0.01% v/v (Sigma-Aldrich). Spraying (0.35 L/min) was carried out with Stihl SR 340 pneumatic spraying equipment (Stihl, Waiblingen, Germany) and was targeted at the lower part of the canopy including the grape area.

- No treatments were carried out on the control vines (Control). Each year, all treatments were harvested on the same day, 22 September in 2011 and 25 September in 2012.

Microvinification

Due to quarantine requirements and logistical issues with shipping frozen grapes from France to Australia, rotundone was analysed indirectly in wines fermented under microvinification conditions. Microscale fermentations were adapted to our experiment according to the protocol proposed by Sampaio et al (2007). This method is known to have low variability and good overall reproducibility. For the rotundone concentration, reproducibility tests showed an average coefficient of variation (CV) of 5% across the micro-fermentors. Each year and for each of the two separate experiments, 15 grape samples were collected at harvest (three per viticultural or maturity treatment). These samples consisted of 600 berries from both sides of the row and from the top (close to the peduncle), central and lower parts of the bunch (25 berries per vine on each side of the row). A sample of berries (800 g) was then weighed (Kern 512 precision balance, Kern & Sohn GmbH, Balingen, Germany) crushed, poured into a 1L Erlenmeyer flask (with lid) and sulfite was added (40 mg/L). After 1 h, the must was inoculated with 200 mg/L rehydrated active dried yeast (522 Davis, Lamothe Abiet, Canejan, France), 300 mg/L of diammonium phosphate added and fermented at 25°C over 8 days. Each fermentation flask was weighed daily to follow the fermentation kinetics and mixed to promote consistent extraction of the rotundone from the berry skins. At the end of the maceration, wines were pressed under controlled conditions (200 kPa for 2 min) using a Para Press laboratory press (Paul Arauner GmbH, Kitzingen, Germany). The amount of wine and of skins was measured to determine skin-to-juice ratio at pressing (%). Wines were then centrifuged (14,000 g for 6 min), received a sulfite addition of 80 mg/L before being bottled and stored at 4°C or below until analysis.

Rotundone analysis

In 2011, rotundone analysis followed the method of Siebert et al. (2008), except for the gas chromatography column, where a DB-35ms (35% phenyldimethyl polysilphenylene-siloxane phase) capillary column of 60 m length, 0.25 mm I.D. and 0.25 µm film thickness (*d*f) was used and the carrier gas (helium) flow rate was 1.4 mL/min in constant flow mode. For the 2012 trial wines, rotundone analysis was again followed the published protocol (Siebert et al. 2008), except multidimensional gas chromatography was used (Figure 1). Multidimensional gas chromatography minimises the possibility of interference from other compounds eluting as adjacent to rotundone. An Agilent Technologies 7890A gas chromatograph (GC) was fitted with a Deans switch microfluidic plate, a flame ionisation detector (FID) and coupled to an Agilent 5975C mass selective detector (MSD). The GC was also equipped with a Gerstel MPS2 XL multi-purpose sampler and cryotrap system (CTS2) (Lasersan Australasia Pty Ltd, Robina, QLD, Australia). The instrument was controlled with Agilent G1701EA ChemStation software in conjunction with Gerstel Maestro software (version 1.4.8.14). The fused silica capillary column set consisted of a VF-35ms (35% phenyldimethyl polysilphenylene-siloxane phase) first dimension (^{1}D) column of 30 m length, 0.25 mm I.D. and 0.25 µm *d*f, with a VF-200ms (trifluoropropyl methyl polysiloxane) second dimension (^{2}D) column of 30 m length, 0.25 mm I.D. and 0.25 µm *d*f, and a restrictor column of 0.7 m length and 0.1 mm I.D. The ^{2}D column was connected with a fused silica universal straight connector (Supelco, Sigma-Aldrich, Sydney, NSW, Australia) to a retention gap of 1 m length and 0.25 mm I.D. which was positioned within the cryotrap. The carrier gas was helium (Ultra High Purity, BOC, Adelaide, SA, Australia), in constant pressure mode with the ^{1}D column head pressure at 320.6 kPa and the ^{2}D column at 241.0 kPa. The Agilent GC split/splitless inlet was fitted with a resilanised borosilicate glass SPME inlet liner (Supelco) and was held at 250°C. A polydimethylsiloxane/divinylbenzene (PDMS/DVB, Supelco) 65 µm SPME fibre was immersed in the SPE extract of a wine sample (Siebert et al. 2008) for 60 min at 35°C, with agitation. The SPME fibre was desorbed in splitless mode and left in the injector for 5 min. The splitter, at 23:1, was opened after 60 sec. The oven temperature was started at 80°C, held at this temperature for 1 min., then increased to 210°C at 5°C/min, cooled to 130°C at 15°C/min and held at 130°C for 3 min, then increased to 210°C at 5°C/min and then increased to 280°C at 20°C/min, and held for 10 min. The MS transfer line was held at 250°C. The cryotrap was cooled to 0°C with liquid N_2 and held at 0°C as the 0.6 min heart-cut from the ^{1}D column was transferred to the retention gap of the ^{2}D column and while the oven was cooled to 130°C, then the cryotrap temperature was increased to 300 °C at 20°C/s and held at 300 °C for 1 min. The MS was operated in positive EI mode at 70 eV with simultaneous selected ion monitoring (SIM) and scanning over a mass acquisition range of 35–280 *m/z ;* for SIM determination of rotundone with d_5-rotundone as internal standard the ions monitored were: *m/z* 147, 161, 163, 203, 208, 218 and 223 dwell time 25 msec each. The target ions were typically *m/z* 218 for rotundone and *m/z* 223 for d_5-rotundone, with the ions 203 and 208 *m/z* used as qualifiers. The data were analysed with Agilent G1701DA ChemStation software.

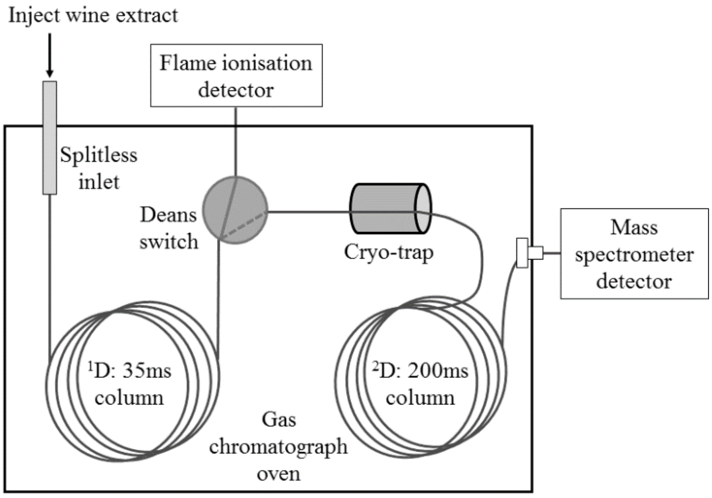

Figure 1. Schematic diagram of multidimensional gas chromatograph (GC) mass spectrometer (MS) for analysis of rotundone in wine.

As with the original protocol, the 2012 multidimensional assay was validated by a series of duplicate standard addition experiments: rotundone was added to a red wine matrix to give concentration values of 0, 2, 5, 11, 20, 51, 100, 199 and 491 ng/L (N = 9 × 2) and then analysed by the method. The precision of the assay was shown by the resultant calibration function being linear throughout the calibration range with excellent correlation (r^2 = 0.999) with a limit of quantitation of 2 ng/L (S/N = 20). The repeatability of the method was also demonstrated by seven replicate additions at a concentration of 16 and 230 ng/L. The relative standard deviation (RSD) was <2% at both of these concentration values. Furthermore, in July 2013, 12 red wine samples from the south-west of France, including seven Duras wines, were analysed by both the original protocol, as in 2011, and the 2012 multidimensional method. A good correlation was observed between the two analyses, r^2 = 0.99. Figure 2 shows a comparison of the chromatograms from the same Duras wine obtained by the two GC methods. Occasionally, compounds co-eluting with rotundone or the internal standard would interfere (as shown in Figure 2a). In MDGC-MS analysis, the second dimension (^{2}D) column re-analyses the heart cut section (Fig. 2b) from the first-dimension column and separates co-eluting compounds from rotundone (Fig. 2c).

As all the experimental wines were made under the same conditions with good reproducibility of the microscale ferments, the concentration of rotundone found in the wines may reflect that of rotundone present in the corresponding berry samples, particularly when a similar sugar content in the grape sample leading to a similar alcohol concentration in wine are observed.

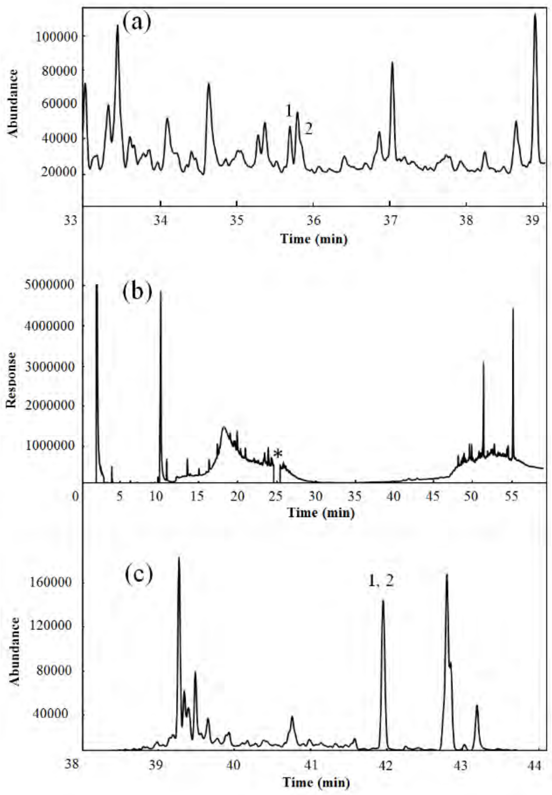

Figure 2. Separation of the volatile components of a Duras wine extract by gas chromatography: (a) partial single-dimension chromatogram (SIM); (b) full ^{1}D chromatogram (FID) showing the heart-cut section marked with an asterisk (*); (c) partial ^{2}D chromatogram (SIM) of the heart-cut components from (b). [1] d_5-rotundone, [2] rotundone.

Grape analysis and δ^{13}C measures

Each year and for each of the two separate experiments, 15 grape samples were collected at harvest (three per viticultural or maturity treatment). Each sample consisted of 216 berries from both sides of the row and several parts of the bunch (9 berries per vine on each side of the row). Berries were then crushed, and the musts obtained were centrifuged (14,000 g for 6 min) to enable classical laboratory and $\delta^{13}C$ analysis. Soluble solids (ºBrix) were measured in duplicate with a digital hand-held Pocket refractometer PAL (Atago, Tokyo, Japan). The remaining amount of centrifuged must was used for $\delta^{13}C$ determination. Tin capsules (TIN 6 x 4 mm) were introduced into a 96-well (8 mm) microplate (model 83.1835; Sarstedt, Nümbrecht, Germany). Five µL of grape juice were introduced in each of the tin capsules using a micropipette. The microplate was then placed in a non-ventilated stove at 60°C for 24 h, closed and sent to the stable isotope laboratory of the James Hutton Institute (Dundee, Scotland). Samples were analysed for their bulk ^{13}C isotopic composition by standard operation procedures compliant with IUPAC guidelines and recommendations for stable isotope ratio measurements and reporting results thereof (Coplen 2011). The precision of the $\delta^{13}C$ values obtained, an integrative indicator of water deficit experienced by vine during grape ripening (van Leeuwen et al. 2009), was determined by repeat analysis of reference materials. Quality control samples were better than ±0.15 %.

Vine measures

For the two experiments, yield at harvest (kg/vine) was monitored for seven out of 12 sample vines by weighing individually crop loads with a Precia Molen C20 K balance (Precia SA, Privas, France). For the experiment on the impact of viticultural techniques, water status of the vine was measured weekly, over the mid-July to end of August period, on the irrigation and control treatments by stem water potential (Ψ_{stem}) according to the protocol defined by Choné et al. (2001). For each experimental unit, measurements were taken from three of the 12 vines and from two mature exposed leaves per vine. These three vines were marked, and stem water potential was always measured on these selected plants. On cloudless days, leaf blades were initially enclosed in plastic bags between 1100 and 1200 h. Measurements took place between 1400 and 1500 h. Pruning wood mass (kg/vine) was measured individually for ten out of

12 vines in January 2012 and 2013 in order to detect any block variability on this parameter; measurements were conducted on the viticultural techniques experiment on 1-year wood.

Statistical analysis

Statistical analyses including linear regressions were conducted with Xlstat software (Addinsoft, Paris, France). All the analytical data and rotundone content of the wines was subjected to a three-way ANOVA treatment (vintage x treatment or picking date x block) with first order interaction (n=30; residual degree of freedom=8). Fisher's least significant difference test was used as a post-hoc comparison of means at $P<0.05$. Before undertaking the linear regressions, a 95% confidence ellipse was applied to the data scatter plots in order to identify and exclude outliers.

Results and discussion

Inter-vintage variability

Results of the three-way ANOVA treatments (Table 1) show that the factor vintage had the strongest overall impact on rotundone concentration with *P values* < 0.0001 for the two experiments. The concentration of rotundone observed in the 2012 vintage was less than half of those in 2011 (Figures 3, 4 and 5). In the south-west of France, the 2011 vintage was characterised by an early budburst, rainfall and high temperature during early vine growth. Flowering occurred 3 weeks earlier than normal. Due to high rainfall during July 2011, however, harvest was advanced only by 7 to 10 days. The 2012 vintage was marked by a severe winter rainfall deficit and low soil moisture at budburst. Despite regular precipitation during the entire growing season, this water deficit was maintained until harvest and led to a protracted veraison, maturation difficulties and variabilities in grape ripeness. Climatic indexes, $\delta^{13}C$ and minimum stem water potential values (Ψ_{stem}) over the mid-July to end of August period presented in Table 2 allow the two vintages to be compared more accurately.

Table 1. Results of three-way ANOVA of rotundone data and other key parameters for the maturity and viticultural techniques trial.

Source of variation	df	*P*-value					
		Rotundone (ng/L)	Sugar content at harvest (°Brix)	$\delta^{13}C$	Skin-to-juice ratio at pressing (%)	Yield at harvest (kg/vine)	Pruning wood mass (kg/vine)
Maturity trial							
Vintage	1	<0.0001	0.152	<0.0001	0.049	0.001	-†
Picking Date	4	<0.001	<0.001	0.726	0.569	0.075	-
Block	2	0.337	0.676	0.140	0.334	0.099	-
Vintage x picking date	4	0.059	0.754	0.270	0.447	0.178	-
Vintage x block	2	0.831	0.387	0.861	0.990	0.127	-
Picking date x block	8	0.580	0.969	0.938	0.994	0.467	-
Viticultural technique trial							
Vintage	1	<0.0001	0.305	<0.0001	<0.0001	0.216	0.015
Treatment	4	<0.001	0.026	0.042	0.019	0.030	0.117
Block	2	0.011	0.033	<0.001	0.157	0.728	0.405
Vintage x treatment	4	0.060	0.288	0.411	0.592	0.995	0.117
Vintage x block	2	0.596	0.092	0.348	0.508	0.850	0.345
Treatment x block	8	0.368	0.541	0.467	0.207	0.630	0.500

† Not determined.

Table 2 shows that vintage in 2011 was warmer than in 2012 over the whole season, including from veraison to harvest. The 2011 vintage, which shows higher rotundone values, was characterised by less water stress (Table 2). Therefore, these results may indicate that summer water stress is a more important parameter than air temperature in explaining differences in rotundone concentration between seasons. These findings do not necessarily contradict the hypothesis that higher a concentration of rotundone is likely to accumulate in cooler vintages (Caputi et al. 2011) and may suggest that rotundone accumulation may be particularly enhanced in vintages with little water stress, i.e. during wetter and cooler vintages. This is in agreement with research work conducted on Mediterranean plants *Pinus halepensis*, *Cistus albidus* and *Quercus coccifera* and particularly *Rosmarinus officinalis,* showing that sesquiterpene leaf emissions were strongly reduced or inhibited after 4 days of water withholding (Ormeño et al. 2007); notably rotundone has been identified in rosemary (Wood et al. 2008).

In the maturity experiment (Table 1), vintage significantly affected grape yield (3.95 kg/vine ± 0.99 in 2011; 2.74 kg/vine ±0.52 in 2012) and skin to juice ratio (24.6% ± 1.6 in 2011; 26.4% +2.3 in 2012). Between the two vintages, changes were also noticed for the viticultural techniques experiment in skin to juice ratio (22.0% ±1.2 in 2011; 26.1% ±1.8 in 2012) and pruning wood mass (0.435 kg/vine ± 0.026 in 2011; 0.490 kg/vine ± 0.090 in 2012). Despite changes in skin-to-juice ratio between vintages (with higher values observed in 2012), significantly higher rotundone concentration was observed in finished wine from 2011, while grape sugar concentration was similar (Table 1). This suggests that differences in grape berry concentration are responsible for differences in rotundone concentration in wine.

Table 2. Characterisation of the 2011 and 2012 vintages by several climatic indexes δ^{13}C and minimum stem water potential (Ψ_{stem}) over the mid-July end of August period values measured on the control treatment of the experimental plot (mean of three replicates).

	Huglin index (IH)	Cool night index (FNv-r)†	Mean air temperature (Tv-r)	Maximal air temperature (Txv-r)	Thermal amplitude (Av-r)	Water stress δ^{13}C	Water stress minimum Ψstem (MPa)
2011	2351	14.7	21.0	28.6	13.9	-25.6 weak‡	-0.97 weak to moderate
2012	2205	14.2	20.5	28.4	14.2	-23.5 weak to moderate	-1.17 moderate to severe

†v-r Veraison–harvest period. ‡Thresholds defined by van Leeuwen et al. (2009).

Effect of grape maturity on the concentration of rotundone in wine

Rotundone concentration measured in the experimental wines is shown in Figure 3. Despite some variability between the experimental plots, no block effect could be identified (*Pvalue*=0.337). In the three-way ANOVA (Table 1), the vintage x picking date *P*-value was close to the 5% threshold, suggesting that the dynamic of accumulation of rotundone during ripening – as reflected by the concentration measured in wine — differed slightly between the two vintages of study. In 2011, the concentration of rotundone in wine from grapes harvested within 7 days between 50% ver. +37 days and 50% ver. +44 days increased rapidly, from a concentration equivalent to its perception threshold to three times this concentration. In this ‘high rotundone’ vintage 2011, rotundone accumulation in the berries of Duras was not slow and gradual. Notably, under the climatic conditions typically found in the south-west of France, the theoretical optimum picking date for Duras is known to occur about 45 days after mid-veraison. In our study, at this time, a plateau in rotundone concentration had been reached, with only a small decrease thereafter. Similar findings have been obtained for related bi- and polycyclic sesquiterpenes in Shiraz and some other cultivars (May and Wüst 2012). In the ‘low rotundone”’ vintage 2012, rotundone accumulation was much slower and similar to previous studies on Vespolina (Caputi et al. 2011) that showed a steady increase in the rotundone content of the berries until 53 days after mid-veraison. In our experiment, an increase was observed across all sampling times. Such inter-vintage difference in the kinetics of rotundone accumulation has recently been described for Shiraz in New Zealand (Logan et al. 2011). In both vintages, the highest rotundone concentration in wine was reached late in ripening, (50% veraison +44 days), which is consistent with studies on Gewürztraminer, which show that expression of some sesquiterpene synthases occurs in the later stages of berry development (Lücker et al. 2004).

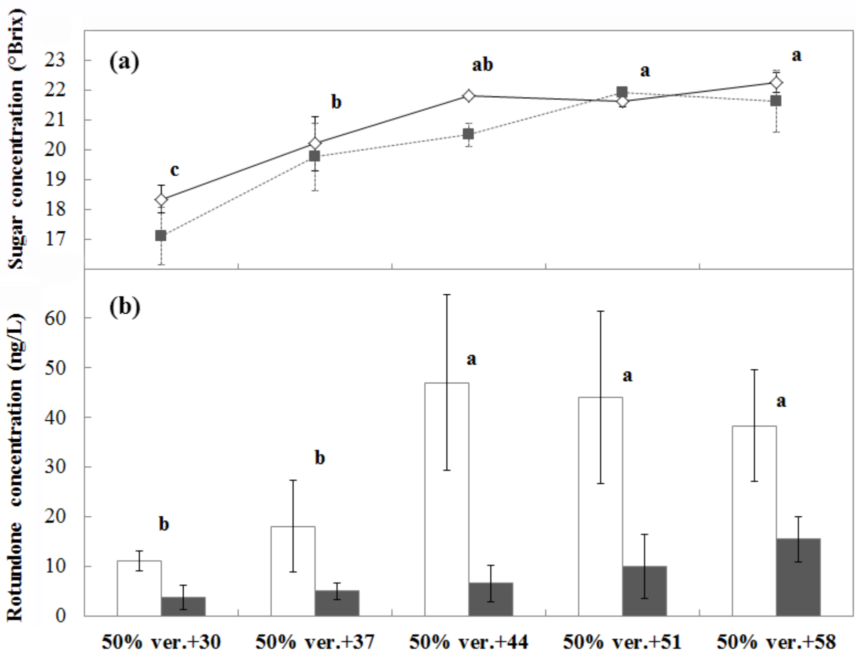

Figure 3. (a) Sugar concentration of the grapes at harvest and (b) rotundone concentration in experimental wines at five levels of maturity. Means of three observations. Error bars refer to inter-block variability. Different letters indicate means significantly different at $P<0.05$ by Fisher test.

Impact of the viticultural techniques on rotundone concentration in wine

Rotundone concentration measured in the experimental wines in response to several viticultural techniques is shown in Figure 4. On this experimental site, a block effect was observed (*P-value*=0.011). The absence of a significant difference in the sugar content between the Control, Irrigation, Elicitor and Leaf treatments (Figure 4) allows a direct comparison of their impact on rotundone concentration in the corresponding wine. Leaf removal at veraison, on both sides of the row, resulted in a significant decrease in the rotundone content of the wines in comparison with that of the control treatment (-69.0% ± 10.3 and -52.4% ± 6.6 in 2011 and 2012, respectively). This decrease suggests that rotundone biosynthesis and/or accumulation in grapes may be affected by incident light intensity and/or the surface temperature of the grapes. The effect of abiotic factors on sesquiterpene emissions from vegetation have been summarised by Duhl et al. (2007): the studies that have examined the light dependency of the emission of sesquiterpenes led to mixed findings, with the majority of studies suggesting that the role of temperature is much more important than light exposure. Spayd et al. (2002) showed that exposing bunches to sunlight could increase berry temperature by as much as 13°C above ambient and the temperature of shaded bunches. Changes to grape surface temperature at the bunch zone is the most likely factor explaining the effect of leaf removal in this study.

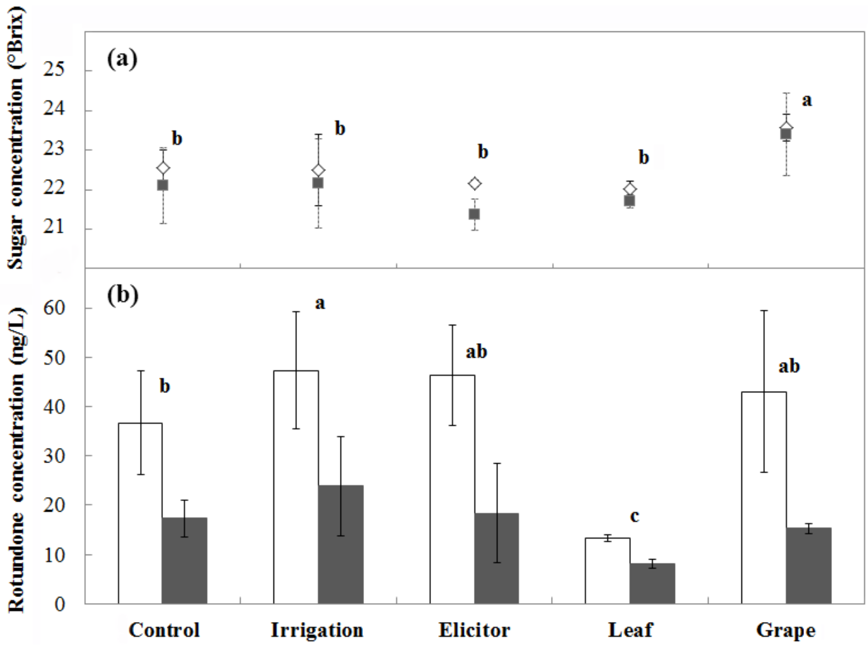

Figure 4. Impact of viticultural techniques on (a) sugar concentration of the grapes at harvest and (b) rotundone concentration in experimental wines in 2011 (◊) and 2012 (■). Means of three observations. Error bars refer to inter-block variability. Different letters indicate means significantly different at *P*<0.05 by Fisher test.

Irrigation significantly modified plant water status in comparison with that of the control. For the irrigated treatment, measured $\delta^{13}C$ (-26.6 ±0.3 in 2011 and -24.3 ±0.7 in 2012) and minimum stem water potential (-0.82 MPa ±0.04 in 2011 and -0.97 MPa ±0.09 in 2012) were significantly lower than that of the control (-25.6 ±1.3 in 2011 and -23.5 ±0.7 in 2012 for $\delta^{13}C$; -0.97 MPa ±0.02 in 2011 and -1.17 MPa ±0.10 in 2012 for minimum stem water potential). Compared to the control, irrigation was the only viticultural treatment showing significant differences in the skin to juice ratio. Despite potential for dilution of compounds from the exocarp due to a significant decrease in this ratio (-8.2 % ±4.1 in 2011; -10.4 % ±3.0 in 2012), rotundone concentration increased on average by 29.1% ± 21.9 in 2011 and by 38.3% ± 23.8 in 2012 in the irrigation treatment in comparison with that of the control. These results are consistent with observed variations in concentration between the vintages discussed previously. They also suggest that rotundone accumulation in berries is linked to the vine water stress deficit over the ten days before the veraison to harvest period and that wetter conditions enhance its biosynthesis. Moreover, grape microclimate may also play a role. Previous studies showed that post veraison irrigation treatments could increase leaf area at harvest (Ginestar et al. 1998). This in turn may lead to a cooler bunch microclimate, promoting the opposite response to that observed during leaf removal and enhancing rotundone accumulation in berries.

With the elicitor treatment, a slight increase in rotundone concentration was observed in 2011, and but not in 2012. No significant difference in the rotundone concentration of the wines was observed compared to that of the control. The efficacy of spraying elicitors is strongly dependent on the conditions of the application, such as concentration of jasmonic acid solution, timing and volume of spraying, and penetration of the canopy. Consequently, it is difficult, based on the results presented here, to draw conclusions about the role of jasmonic acid.

The grape thinning treatment was the only treatment that caused a significant difference in yield at harvest. A crop load of 2.33 ±0.70 and 1.88 ±0.48 kg/vine was recorded in 2011 and in 2012, respectively, whereas yield reached 3.73 ±0.40 and 3.19 kg/vine ±0.14 for the control treatment. Bunch number averaged 18.3 ±1.4 and 13.5 ±0.5 for the control treatment in 2011 and in 2012, respectively. These averages were reduced to 10.9 ±1.9 and

7.9±1.4 for the grape thinning treatment. As could be expected (Dokoozlian and Hirschfelt 1995), berry sugar concentration at harvest was highest in the grape thinning treatment (Figure 4). Despite this leading to higher alcohol in wine (Figure 4), which favours extraction, no significant difference was observed for wine rotundone concentration between the grape thinning and control treatments.

Intra-plot variability and correlation between rotundone concentration of wines and $\delta^{13}C$

Analysis of the viticultural experimental treatments showed a strong block effect. The block located in the lower part of the site (foothill) had a higher concentration of rotundone in comparison with that of the blocks located on the hillside and top of the site (Figure 5).

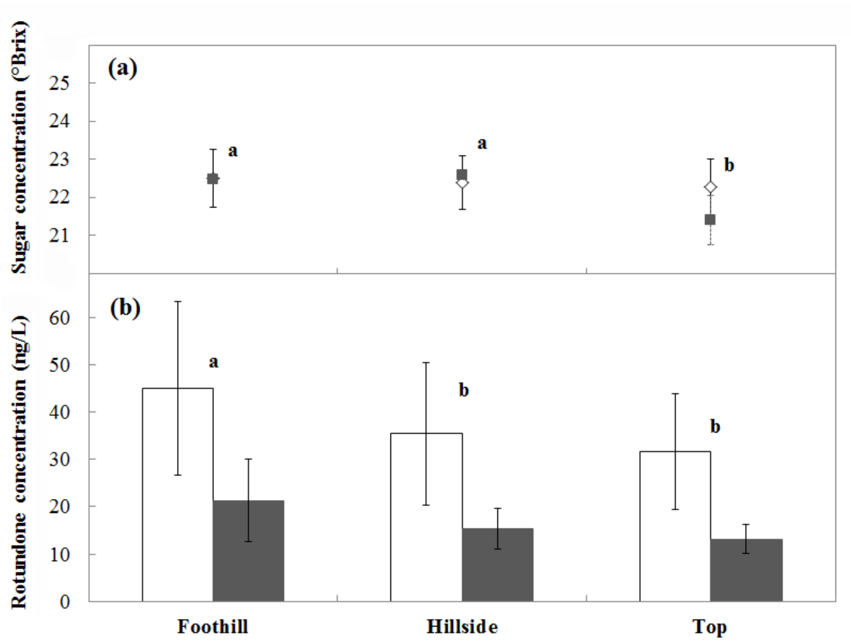

Figure 5. (a) Sugar concentration of the grapes at harvest and (b) rotundone concentration in experimental wines from the three blocks of the experimental site where the viticultural techniques were undertaken in 2011 (◊) and 2012 (■). Means of five observations. Error bars refer to inter-treatment variability. Different letters indicate means significantly different at *P*<0.05 by Fisher test.

Other research has observed a higher rotundone concentration in grapes collected from hillsides rather than from planes (Caputi et al. 2011). It may be, however, that 'location' is not a causative factor as such, but may be confounding the effect of other variables previously discussed: across all the major parameters measured on grapes at harvest and on vine, sugar content and $\delta^{13}C$ are the two variables that were significantly affected by 'block' factor (Table 1). Among these two parameters, only $\delta^{13}C$ significantly discriminates the foothill block ($\delta^{13}C$= -27.0 ±0.1 in 2011; $\delta^{13}C$= -24.6 ±0.4 in 2012) from the hillside block ($\delta^{13}C$= -25.9 ±0.9 in 2011; $\delta^{13}C$= -23.5 ±0.3 in 2012) and top block ($\delta^{13}C$= -26.2 ±0.2 in 2011; $\delta^{13}C$= -23.3±0.5 in 2012). Therefore, $\delta^{13}C$, a proxy for vine water status, may be the key measurement explaining the variability in the rotundone concentration observed within the experimental site. Correlations with levels of significance below 0.0001 in 2011 and below 0.001 in 2012 (Figure 6) were observed between rotundone concentration in wine and $\delta^{13}C$ measured on grape sugar at harvest on the data of the viticultural techniques site (r^2=0.76 in 2011 and r^2=0.74 in 2012). Leaf removal values were excluded from the treatment. The measure of $\delta^{13}C$ could assist in the identification of regions of water deficit within a vineyard, which would facilitate the identification of zones for selective harvesting to produce grapes with a desired concentration of rotundone and thereby produce wines with the desired level of black pepper aroma.

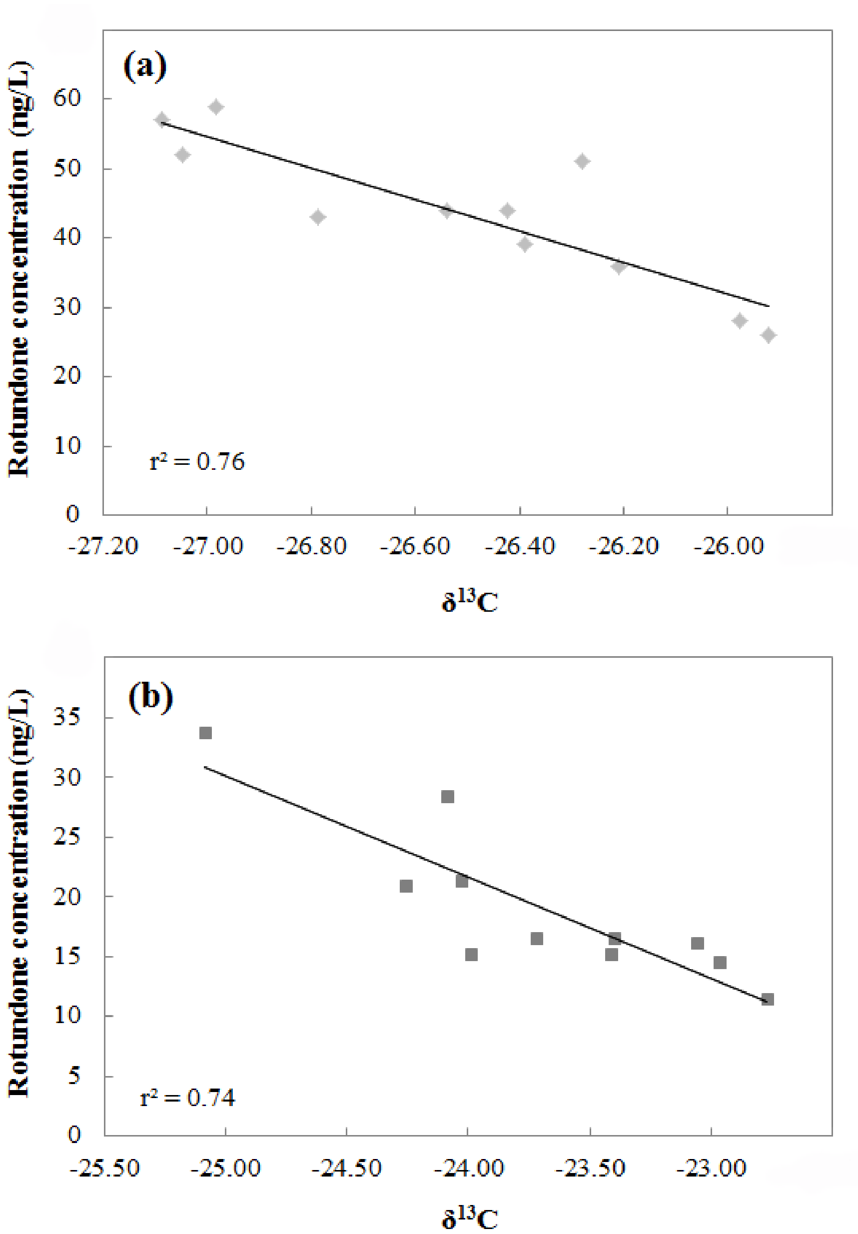

Figure 6. Relationship between rotundone concentration in wines and $\delta^{13}C$ measured on the fruit sugar at harvest on the experimental site where the viticulture techniques were undertaken in (a) 2011 and (b) 2012. Data did not include leaf removal values. In 2011 and 2012, one outlier located outside the 95% confidence ellipse of the data scatter plots, was removed from the treatment. Linear model gave (a) y = -22.795x – 560.82; r^2 = 0.76 and (b) y = -8.4543x – 181.33; r2 = 0.74.

Conclusion

This study has enabled a better understanding of some viticultural aspects that affect rotundone concentration in the cv. Duras. Micro-vinification of individual grape samples showed that wine rotundone concentration was a proxy for grape rotundone concentration when no significant difference in sugar content, and subsequent alcohol concentration in wine, were observed. We showed that the accumulation of rotundone during ripening was dependent on the vintage conditions, and that the highest concentration of rotundone was reached late in the berry development from 44 days after mid-veraison. Application of exogenous jasmonic acid did not significantly increase rotundone concentration in this study. Crop load reduction did not significantly affect rotundone concentration, whereas leaf removal reduced rotundone concentration in the resulting wine. These findings suggest that rotundone biosynthesis is affected by light exposure in the bunch zone and/or by its consequent effect on the surface temperature of the grapes. Wines from the irrigated treatment showed a higher concentration of rotundone and more generally, vine water status, over the period from veraison to harvest (as measured by $\delta^{13}C$ values) was a key parameter that correlated with intra-plot variability. On the experimental site, correlations (r^2=0.76 in 2011 and r^2=0.74 in 2012) were observed between the rotundone concentration in experimental wines (ng/L) and $\delta^{13}C$ values measured of fruit sugar at harvest. These findings identify practical opportunities to manipulate rotundone content and the peppery character of Duras wines through leaf removal, irrigation and selective harvest coupled with water-deficit mapping. The results obtained may also be applicable to grapegrowers producing Syrah, Pineau d'Aunis, Gamay and Graciano, or other cvs in which rotundone has been identified.

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Article 6:

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Using common viticultural practices to modulate the rotundone and 3-isobutyl-2-methoxypyrazine composition of *Vitis vinifera* L. cv. Fer N red wines from a temperate climate wine region with very cool nights

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Résumé : Ces travaux visaient à étudier l'impact de trois pratiques viticoles sur la concentration en 3-isobutyl-2-methoxypyrazine (IBMP) et en rotundone des vins de Fer N de l'AOP Marcillac, dans l'objectif d'orienter leur profil sensoriel vers davantage de notes poivrées. Si l'effeuillage n'a eu aucun impact significatif sur les deux composés, l'échardage a permis une réduction significative de la teneur des vins en IBMP. La vendange différée, 7 jours après le témoin, apparaît la pratique la plus recommandable puisqu'elle a induit un gain en rotundone associé à une baisse en IBMP.

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Using common viticultural practices to modulate the rotundone and 3-isobutyl-2-methoxypyrazine composition of *Vitis vinifera* L. cv. Fer red wines from a temperate climate wine region with very cool nights

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ABSTRACT

Aim: Rotundone and 3-isobutyl-2-methoxypyrazine (IBMP) are two potent aroma compounds responsible for pepper and bell pepper notes in red wines, respectively. The aim of the study was to modulate, through common viticultural practices, the volatile composition in these two molecules of Fer red wines from a temperate climate wine region with very cool nights, located in the southwest of France.
Methods and results: Three viticultural practices (leaf removal 10 days after berry set, removal of lateral shoots, and delayed harvest 7 days after the control) were investigated in 2015 and in 2016. Rotundone concentrations up to 69 ng/L were found in experimental wines. IBMP concentrations were below perception level in wines from 2016 and below detection level in wines from 2015, a vintage with particularly hot climatic conditions between berry set and bunch closure. Delayed harvest induced an increase in rotundone concentration while leaf removal and the removal of lateral shoots had no significant impact on rotundone concentration. Delayed harvest and the removal of lateral shoots were the most efficient practices to decrease IBMP in wines. The three techniques made it possible to increase the odour activity values (OAV) ratio of OAV rotundone to OAV IBMP, with the greatest impact observed for delayed harvest.
Conclusion: According to our results, delayed harvest appears to be the best practice to modulate the volatile composition of Fer wines toward an increase in the OAV rotundone to OAV IBMP ratio.
Significance and impact of the study: Our results may assist local grape growers to modulate the volatile composition of their wines.

KEYWORDS

IBMP, rotundone, leaf removal, removal of lateral shoots, delayed harvest

INTRODUCTION

Vitis vinifera L. cv. Fer is a dark-skinned grape variety which in 2015 covered 1548 ha exclusively in the southwest of France, according to FranceAgriMer (www.franceagrimer.fr). Together with Merlot, Cabernet Sauvignon, Cabernet Franc, Carmenère, Petit Verdot, Castets and other minor cultivars, this variety was considered part of the ecogeographic group named "carmenets" by Bisson (1995, following Levadoux, 1948), according to morphological traits and supposed geographical origin. The name Fer is derived from the latin *ferus,* meaning 'wild, savage' which is consistent with the common belief that Fer was domesticated from local wild grapevines (Lavignac and Audiot, 2001). It has several direct offspring belonging to the ancient southwest of France varietal heritage, proof of its seniority and its wide distribution in the vineyards of this part of France. For example, DNA parentage analysis indicated that Fer was related to Mauzac Noir du Lot-et-Garonne (which is not a colour mutation of Mauzac) and birthed Négret de Banhars and Gros Cabernet through spontaneous crossings with Manseng Noir and Txakoli, respectively (Lacombe *et al.*, 2013). Thus, Fer is one of the grandparents of Carmenère, which is an offspring of Gros Cabernet and Cabernet Franc (Boursiquot *et al.*, 2009). The earliest literary references to Fer appeared in 1783 (Rézeau, 1998) and this variety is presently known under several synonyms (i.e Braucol in Gaillac, Servadou in Lot-et-Garonne, Pinenc in Saint-Mont or Madiran, Mansois in Marcillac). More recently in 1966, Fer was used by INRA to breed Ferradou with Merlot (plantgrape.plantnet-project.org).

Fer gives medium bodied wines which often exhibit bell-pepper-like odours associated with substantial levels of 3-isobutyl-2-methoxypyrazine (IBMP) (Davaux, 2005). This attribute is particularly enhanced when grapes are not sufficiently ripe or are produced under cool and wet climate conditions (Roujou de Boubée, 2000). Notably with concentrations up to 110 ng/L (Davaux, 2005), IBMP has been identified as an aromatic marker of Fer wines from the Protected Designation of Origin (PDO) Marcillac in the French Aveyron department, which is locally considered as a cool climate vineyard. Fer wines from this wine region can also develop some distinctive peppery notes that are attributable to rotundone, a sesquiterpene discovered in 2008 (Wood *et al.*, 2008), with concentrations up to 18 ng/L (Olivier Geffroy, unpubl. data, 2014). IBMP and rotundone are among the most potent aroma compounds identified in wine with detection thresholds in red wine of 15 ng/L and 16 ng/L, respectively (Roujou de Boubée, 2000; Wood *et al.*, 2008). Both molecules have been described by Ferreira (2012) as two of the 16 most impactful aroma compounds in wine that can break its "buffer" without the support of additional odorants.

In addition to Fer, IBMP has been identified in cultivars mainly from the Carmenets group such as Sauvignon, Cabernet Sauvignon, Merlot and Cabernet Franc (Allen *et al.*, 1995; Hashizume and Umeda 1996). Rotundone has been found in in a wide range of varieties, including Pinot Noir, Durif, Graciano and Riesling (Herderich *et al.*, 2012), Duras (Geffroy *et al.*, 2014), Gamay (Geffroy *et al.*, 2016a), Malbec and Abouriou (Cullere *et al.*, 2016) and Vespolina, Schioppettino and Grüner Veltliner (Caputi *et al.*, 2011).

While IBMP is usually considered by wine professionals as an undesirable aroma compound in red wine, rotundone is generally positively perceived by consumers (Geffroy *et al.*, 2018). The modulation of the sensory profile of Marcillac red wines toward a spicier character is an issue frequently raised by local winemakers. This modulation, which may involve an increase in rotundone concentration coupled with a decrease in IBMP concentration, can be challenging. Indeed, rotundone and IBMP are produced *in situ* and are not sensitive to source-think variations (Geffroy *et al.*, 2016; Koch *et al.*, 2010; Zhang *et al.*, 2016). In addition, both compounds are generally enhanced by cool and wet climatic conditions (Caputi *et al.*, 2011; Mendez-Costabel *et al.*, 2013).

However, some viticultural practices could be valuable to reach this objective. Indeed, the kinetic of accumulation during grape maturation differs between IBMP and rotundone. The bell pepper aroma compound is produced between berry set and bunch closure and its concentration in berries decreases during grape ripening (Roujou de Boubée *et al.*, 2000; Ryona *et al.*, 2010). Rotundone starts its accumulation from mid-veraison, and the highest rotundone concentrations are reached late in the berry development (Caputi *et al.*, 2011; Geffroy *et al.*, 2014).

In the same way, distinct responses to canopy management techniques and notably defoliation have been observed for the two aroma compounds. It has been shown that an early leaf removal, especially just after berry set (Roujou de Boubée, 2000; Davaux, 2005; Scheiner *et al.*, 2010; Šuklje *et al.*, 2012, Sivilotti *et al.*, 2016), and the removal of lateral shoots (Roujou de Boubée, 2000) were effective to reduce IBMP in berries and wines. The effects of sunlight and temperature on fruit composition induced by defoliation are always difficult to separate, as many biochemical pathways are affected by these two abiotic factors. The most likely hypothesis is that bunch light exposure induces a photochemical degradation reaction of IBMP that could be affected secondarily by the increase in temperature (Šuklje *et al.*, 2012). Conflicting results have been found concerning the impact of leaf removal on rotundone: leaf removal can either reduce, increase or have no impact at all on rotundone concentrations (Geffroy *et al.*, 2014; Homich *et al.*, 2017) depending on its timing, its intensity and the site. The impact of the removal of lateral shoots on rotundone concentration has not been yet investigated.

In 2015 and 2016, three viticultural practices (leaf removal 10 days after berry set, removal of lateral shoots and delayed harvest 7 days after the control) were investigated in a field trial to modulate the volatile composition of Fer wines from the Marcillac PDO toward an increase in the OAV rotundone to OAV IBMP ratio.

MATERIAL AND METHODS

1. Experimental design

The experiment was established on a 0.80-ha vineyard located within the Marcillac PDO (latitude 44° 27′ 5″′ N; longitude 02° 25′ 43″′ E) with a slope of 15%. This vineyard typical of the area with 2.20 m × 1 m vine spacing and vines was trained with vertical shoot positioning on a single Guyot pruning system. The soil was managed by chemical weed control under the vines and by grass cover in every inter-row. The date of establishment of the site and the rootstock were not determined because the vineyard was planted more than 50 years ago. The trial was conducted on four rows in a randomised complete block design with three replications per treatment. Each experimental unit of 26.4 m^2 contained 12 continuous vines. Orientation of the vine rows was north-west to south-east.

2. Viticultural practices

Three viticultural techniques were investigated and compared with a control treatment.

- 100% fruit zone leaves (leaf removal) were removed by hand on the east-facing side 10 days after berry set, on 17 June in 2015 and on 7 July in 2016. All leaves were removed from the basal node to the first node above the top bunch.

- Lateral shoots were removed manually twice (removal of lateral shoots). The first operation was undertaken at berry set on 7 June in 2015 and on 27 June in 2016 and the second one three weeks later, on 28 June in 2015 and on 3 July in 2016.

- For the delayed harvest treatment (delayed harvest), grapes were harvested exactly 7 days after the control and the two other studied treatments on 5 October in 2015 and on 26 October in 2016.

3. Vine and weather measurements

Harvest, except for the delayed harvest treatment, took place on 28 September in 2015 and on 19 October in 2016, which corresponds exactly to 54 days after mid-veraison. Yield (kg/vine) was monitored for each vine by weighing individually crop load with a Precia Molen C20 K balance (Precia SA, Privas, France). In 2015 and in 2016, nine temperature loggers (EBI 20-T1, Ebro Electronic, Germany) displayed in solar radiation shields were positioned in the bunch zone for each experimental unit except for the delayed harvest treatment. These data were used to study the impact of the techniques on bunch microclimate through calculation of mean air temperature (Tv-r), cool night index (FNv-r) and maximal air temperature (Txv-r) between veraison and harvest (Tonietto and Carbonneau, 2002). In 2015, bunch surface temperature was identified as one of the main determinants of rotundone, which could be predicted in grapes using the percentage of degree hours above 25°C (DH25) (Zhang *et al.*, 2015). Consequently, DH25 was calculated using the data from the temperature loggers, and complementary measurements of bunch surface temperature were undertaken in 2016 using a non-contact infrared thermometer. These measurements were carried out for the control, leaf removal and removal of lateral shoots treatments between mid-veraison and harvest on 21 September 2016, a cloudless and windless day. Bunch surface temperature was determined halfway between the two end points of the bunch using a non-contact Raynger MX infrared thermometer (Raytek, USA). Measurements were made on 30 bunches for each experimental unit on each side of the vine at 12:00 h and 16:00 h. The measurements in the experimental plot were completed in less than 15 min for each time of measurement. Bunch surface temperature was first to be assessed for all the experimental units on the east-facing side and then on the west-facing side of the row. The same day, the opportunity was taken to determine stem water potentials (Ψ stem) and leaf nitrogen status. For each experimental unit, nitrogen status was characterised on 12 leaves (one leaf per vine) through measurements of Dualex® (Force A, Orsay, France), a fluorescence-based sensor that allows the calculation of a chlorophyll to flavonols ratio named NBI® (nitrogen balance index). Stem water potentials were measured according to the method proposed by Choné *et al.* (2001). Measurements were taken from three of the 12 vines and from two mature exposed leaves per vine. Leaf blades were initially enclosed in plastic bags between 11:00 h and 12:00 h. Measurements were made between 14:00 h and 15:00 h. Since 2005, rainfall and air temperature (minima, maxima and mean values) have been monitored by a CimAGRO weather station (Cimel Electronique, Paris, France) placed within 5 km of the experimental site. These data were used to characterise the climate of the area for the 2005–2014 period and the two vintages of study through calculation of Huglin index (Huglin, 1986) and cumulative rainfall. Cumulative rainfall between veraison and harvest were not determined for the 2005–2014 period as veraison dates were unknown. The time necessary to remove fruit zone leaves and lateral shoots was also assessed for each experimental unit.

4. Berry sampling and analyses

At harvest for each experimental site, two samples consisting of 200 berries and 800 g of berries were collected on each experimental site. The 200-berry sample was weighted, crushed gently, and the musts obtained were centrifuged to enable traditional laboratory measurements. Sugar concentration (°Brix) was determined with a digital hand-held Pocket refractometer PAL (Atago, Japan). Titratable acidity was measured according to the Organisation Internationale de la Vigne et du Vin (OIV) method (OIV 2009) and pH was measured with a HI 3221 pH meter (Hana Instruments, France). A Konelab Arena 20 sequential analyzer (Thermo Electron Corporation, USA) associated with enzyme kits provided by several suppliers was used to determine amino acids, ammonium (Megazyme, Ireland) and malic acid (Thermo Fisher Scientific, USA). Potassium determination was carried out by flame photometry (Bio Arrow, France) according to OIV method (OIV 2009) and tartaric acid determination by colorimetric titration (Hill and Caputi, 1970). $\delta^{13}C$ based upon $^{13}C/^{12}C$ ratio was determined at harvest on grape sugars for each experimental unit according to a published protocol (Geffroy *et al.* 2014). In water stress conditions, sugars contain more ^{13}C compared to those produced when the water status is not limiting, and this isotopic ratio can be used as an integrative indicator of water deficit (van Leeuwen *et al.*, 2009) experienced by vine during grape ripening. The remaining berry skins were crushed for 2 minutes at low speed (600 rpm) using a food blender (Moulinex, Faciclic, France) to perform phenolic analyses. Anthocyanins and total phenolic index (TPI) were quantified in grapes according to the method described by Cayla *et al.* (2002), which can be summarised here briefly. 50 g of the crushed berries were macerated for 1 h in a medium containing 15 mL of ethanol (95%) and 85 mL of HCl (37%). Samples were stirred every 15 minutes and after centrifugation (14 000g for 6 min), anthocyanins and TPI were quantified the same way as in wine according to Ribéreau-Gayon and Stonestreet (1965) and Ribéreau-Gayon (1970), respectively, using an Evolution 100 spectrophotometer (Thermo Electron Corporation, USA).

The 800 g grape samples were used to perform microscale fermentations in 1 L Erlenmeyer flasks according to the protocol proposed by Geffroy *et al.* (2014). At the end of the 8-day maceration at 25°C, wines were pressed under controlled conditions (200 kPa for 2 min) using a Para Press laboratory press (Paul Arauner GmbH, Kitzingen, Germany). Wines were then centrifuged (14 000 × g for 6 min), received a sulfite addition of 80 mg/L before being bottled and stored at 4°C or below. After 1 month and as part of a contract service, rotundone and IBMP were determined in wines by the AWRI (Adelaide, Australia) with SPME-GC-MS using deuterated isotopes and parameters previously described (Geffroy *et al.*, 2014; King *et al.*, 2011). Reproducibility tests showed an average coefficient of variation of 5% across the microfermentors for the rotundone and IBMP concentrations. Limits of quantification were 2 ng/L and 5 ng/L for rotundone and IBMP, respectively. Odour activity values (OAVs) determined by dividing the concentrations of the two odorants by their perception thresholds in wine were used to calculate the OAV rotundone to OAV IBMP ratio.

5. Data treatment

Statistical analyses were conducted with Xlstat software (Addinsoft, France). All the analytical data measured both in 2015 and in 2016 that included rotundone were subjected to a three-way analysis of variance (ANOVA) treatment (vintage × treatment × block) with first-order interaction. Other variables which were just determined, detected or calculated during one year of study (i.e. IBMP, ratio of OAV rotundone to OAV IBMP) were treated by a two-way ANOVA (treatment × block) with or without first-order interaction depending if measurements were repeated for each experimental unit. Bunch surface temperature data were subjected to a four-way ANOVA (treatment × side of the row × time of measurement x block) with first-order interaction. Fisher's least significant difference (LSD) test was used as a post-hoc comparison of means at $P < 0.05$.

RESULTS AND DISCUSSION

1. Effect of the vintage

Results of the ANOVA treatments showed that among the studied factors the vintage had the greatest impact on the measured variables (Table 1). For most of the variables, no block effect or significant interactions between the factors were observed. However, for ammonium, potassium and anthocyanins, significant interactions were found, involving the vintage factor and/or the block factor and the treatment factor. This indicates that the impact of the studied techniques can differ notably according to the characteristics of the vintage.

Climatic data presented in Table 2 and Table 3 allow us to characterise the climate of the region and the two vintages of the study.

According to Tonietto and Carbonneau (2002), the climate of the Marcillac PDO can be considered as temperate with very cool nights.

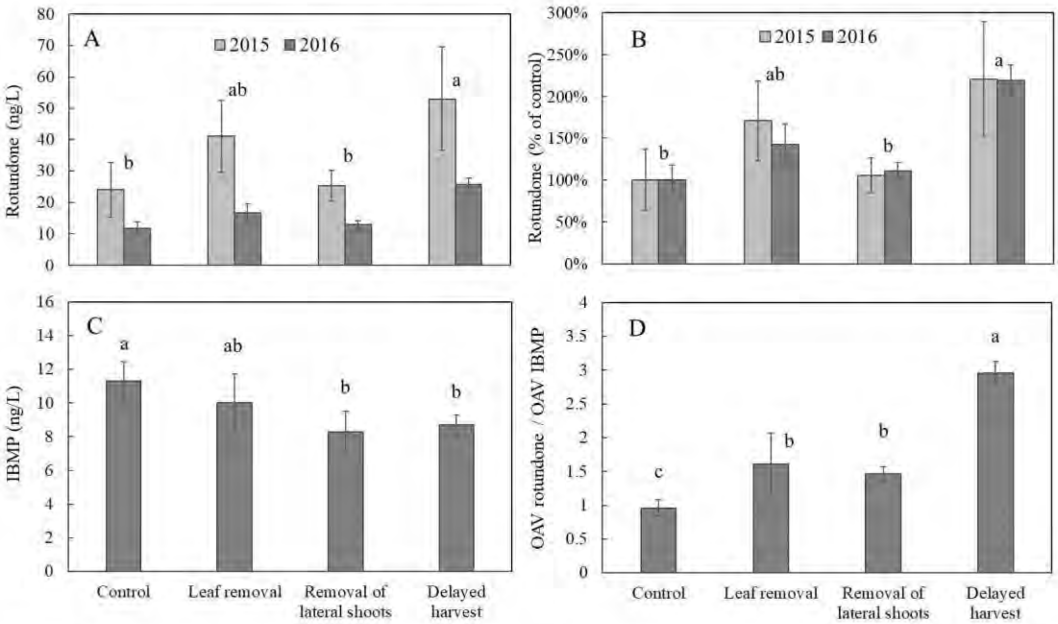
TABLE 1. Results of the ANOVA performed on the variables measured during the trial.

Variable	*P*-value					
	Vintage (V)	Treatment (T)	Block (B)	V × T	V × B	T × B
Mass of 200 berries	0.102	0.341	0.900	0.410	0.559	0.137
Sugar concentration	< 0.001	0.542	0.718	0.903	0.412	0.849
Titratable acidity	< 0.001	0.168	0.204	0.218	0.626	0.479
pH	< 0.001	0.169	0.319	0.283	0.349	0.136
Malic acid	< 0.001	0.183	0.100	0.084	0.486	0.077
Tartaric acid	0.672	0.290	0.761	0.152	0.802	0.492
Amino acids	0.005	0.005	0.320	0.548	0.510	0.219
Ammonium	0.004	0.004	0.009	0.020	0.233	0.004
Potassium	0.001	0.009	0.356	0.040	0.165	0.964
Anthocyanins	< 0.001	0.001	0.724	0.072	0.029	0.011
Total phenolic index (TPI)	< 0.001	0.076	0.834	0.647	0.358	0.551
Yield at harvest	< 0.001	0.313	0.090	0.878	0.207	0.477
Mean air temperature (Tv-r)	0.227	0.798	0.815	0.999	0.785	0.985
Cool night index (FNv-r)	0.011	0.521	0.521	0.396	0.769	0.644
Maximal air temperature (Txv-r)	0.038	0.981	0.877	0.678	0.939	0.199
DH_{25}	0.003	0.713	0.433	0.402	0.876	0.692
$\delta_{13}C$	0.776	0.027	0.393	0.500	0.858	0.306
Stem water potential (Ψ_{stem})	n.d.	0.009	0.003	n.d.	n.d.	0.064
Nitrogen balance index (NBI)	n.d.	0.205	0.522	n.d.	n.d.	0.069
Rotundone	<0.001	0.002	0.403	0.310	0.498	0.455
2-methoxy-3-isobutylpyrazine	n.d.	0.060	0.736	n.d.	n.d.	n.d.
OAV of rotundone to OAV of IBMP ratio	n.d.	<0.001	0.086	n.d.	n.d.	n.d.

TABLE 2. Characterisation of the area's climate and the two vintages by Huglin index and cumulative rainfall.

		Cumulative rainfall (mm)		
Vintage	Huglin index (HI)	1 Jan – 31 Dec	1 Apr – 30 Sep	Veraison – Harvest
2005–2014	1838	804	425	n.d.
2015	2031	736	367	164
2016	1871	1107	525	123

FIGURE 1. Effect of the techniques on A) rotundone expressed in ng/L in 2015 and in 2016; B) rotundone expressed in percentage of control in 2015 and in 2016; C) IBMP in 2016; and D) the ratio of OAV rotundone to OAV IBMP in 2016 (mean of three observations). a–d indicate statistically significant differences at $P<0.05$ except for IBMP ($P=0.06$).



The 2015 vintage was characterised by limited rainfall events and hot weather conditions over the whole vine vegetative cycle. The beginning of the 2015 summer was one of the warmest ever recorded in the area, with two heat waves occurring in June and in July. During these two extreme climatic events, maximum temperature exceeded 40°C for several days. Frequent thunderstorms and rain showers were observed during the month of August, which was marked by cooler and wetter climatic conditions. In comparison with 2015, the 2016 vintage (which remains a hot vintage) was cooler and rainier, as reflected by Huglin index and cumulative rainfall. Despite rainy and cool weather conditions from spring to the beginning of summer, which induced a late-flowering, an outstanding flowering and berry-set rate were observed that lead to high yield at harvest for the area. Grapevine yield formation extends over two consecutive seasons. The formation of inflorescence primordia that occurs in the first season, typically in June under our experimental conditions, is enhanced by temperature and light (Srinivasan and Mullins, 1981). We can assume that the particularly hot and sunny conditions observed in June 2015 might explain the large crop level obtained at harvest in 2016, the second season. Conditions were hot and dry in August and throughout the maturation in 2016. The delay in phenological development observed at flowering was maintained until harvest, which occurred 3 weeks later than in 2015.

TABLE 3. Effect of the studied techniques on the variables for which no significant interaction involving the treatment factor was observed (mean and standard deviation of three observations)

Variable	Control			Leaf removal			Removal of lateral shoots			Delayed harvest		
	2015	2016		2015	2016		2015	2016		2015	2016	
Mass of 200 berries (g)	283±11	302±24	a	267±19	292±16	a	287±13	284±18	a	292±22	297±13	a
Sugar concentration (°Brix)	22.2±0.8	19.4±1.0	a	22.6±0.4	19.7±1.2	a	20.8±0.4	19.2±0.8	a	22.8±0.8	19.4±0.4	a
Titratable acidity (g/L of tartaric acid)	7.08±0.11	8.20±0.19	a	7.38±0.19	8.86±0.42	a	7.12±0.19	8.83±0.30	a	7.36±0.24	8.17±0.25	a
pH	3.14±0.02	3.05±0.03	a	3.14±0.03	3.02±0.04	a	3.17±0.01	3.02±0.03	a	3.17±0.03	3.06±0.05	a
Malic acid (g/L)	1.35±0.08	2.26±0.14	b	1.40±0.13	2.44±0.13	a	1.37±0.08	2.41±0.26	ab	1.48±0.08	2.25±0.11	ab
Tartaric acid (g/L)	5.05±0.30	4.57±0.17	a	4.89±0.05	5.49±0.51	a	5.35±0.21	4.92±0.07	a	4.34±0.02	5.01±0.75	a
Amino acids (mg/L)	46.1±2.1	61.1±6.8	b	46.1±8.2	54.7±9.7	b	51.2±5.2	57.4±2.4	b	63.9±3.4	71.9±6.8	a
Total phenolic index (TPI)	59±1	96±3	a	68±5	103±10	a	62±7	102±3	a	59±6	91±4	a
Yield at harvest (kg/vine)	2.24±1.00	2.84±1.11	a	1.95±1.06	2.71±1.74	a	2.26±1.17	2.64±0.82	a	2.46±1.02	3.16±1.72	a
Mean air temperature (Tv-r)	18.09±0.54	18.43±0.25	a	18.22±0.08	18.58±0.16	a	18.06±0.15	18.40±0.15	a	n.d.	n.d.	
Cool night index (FNv-r)	11.02±0.24	9.24±0.26	a	10.24±0.95	9.29±0.16	a	10.90±0.16	9.12±0.06	a	n.d.	n.d.	
Maximal air temperature (Txv-r)	29.17±2.94	27.02±0.89	a	30.10±0.78	26.22±1.00	a	29.49±2.77	27.33±1.18	a	n.d.	n.d.	
$DH_{25}{}^{a}$	3.31±0.38	2.42±0.14	a	3.64±0.08	2.32±0.24	a	3.45±0.03	2.42±0.10	a	n.d.	n.d.	
$\delta_{13}C$	-26.69±0.16	-26.41±0.34	a	-26.99±0.16	-26.89±0.40	ab	-26.85±0.07	-26.76±0.61	a	-27.19±0.26	-27.49±0.37	b
Stem water potential (MPa)	n.d.	-0.57±0.10	b	n.d.	-0.50±0.09	a	n.d.	-0.47±0.06	a	n.d.	n.d.	
Nitrogen balance index (NBI)	n.d.	5.43±0.98	a	n.d.	5.24±1.19	a	n.d.	5.69±0.80	a	n.d.	n.d.	

TABLE 4. Effect of the studied techniques on the variables for which at least one significant interaction involving the treatment factor was observed.

Variable	Control			Leaf removal			Removal of lateral shoots			Delayed harvest		
	Block			Block			Block			Block		
	1	2	3	1	2	3	1	2	3	1	2	3
2015												
Ammonium (mg/L)	44.6	41.8	45.6	22.1	25.4	60.1	53.1	55.6	50.0	49.3	58.0	63.1
Potassium (g/L)	0.85	0.76	0.85	0.91	0.85	0.90	0.91	0.83	0.82	0.60	0.60	0.68
Anthocyanins (mg/kg)	1090	1066	1160	1304	1347	1224	1241	1003	1065	1091	1007	949
2016												
Ammonium (mg/L)	43.1	48.4	45.1	29.5	21.5	51.5	37.0	40.3	33.8	35.2	52.0	40.7
Potassium (g/L)	0.87	0.94	0.82	1.02	1.01	1.01	0.99	0.95	0.93	1.01	0.96	0.81
Anthocyanins (mg/kg)	1474	1528	1633	1451	1730	1588	1549	1483	1581	1490	1394	1394

The effect of the studied factors on classical enological parameters, yield at harvest, leaf nitrogen status, water status and bunch microclimate, is summarised in Tables 3 and 4. IBMP and rotundone concentrations as well as the ratio of OAV rotundone to OAV IBMP, are shown in Figure 1 (A–D).

No differences in mean air temperature between veraison and harvest were observed between the two vintages, but 2015 was characterised by a greater cool night index, maximal air temperature and percentage of degree hours above 25 °C between veraison and harvest. These climatic features lead to lower concentration in malic acid and titratable acidity, and a higher pH at harvest in 2015 in accordance with previous findings (Coombe, 1987). In 2016, the larger yields observed and the late harvest date lead to a lower sugar concentration at harvest (Kliewer and Dokoozlian, 2005). While higher crop load should have negatively impacted the accumulation of phenolic compounds in grapes (Kok, 2016), anthocyanins and TPI were surprisingly greater for this vintage. We can suppose that the elevated day temperature conditions experienced in 2015 contributed to decrease anthocyanins content, whose degradation is promoted by temperatures above 30°C (Mori *et al.*, 2007). Cooler nights might have also enhanced anthocyanins synthesis in berries in 2016 (Kliewer and Torres, 1972). The reasons why TPI was superior in 2016 remain unclear as the total levels of proanthocyanidins, which can be considered as the main contributor to TPI (Blouin *et al.*, 2000), are weakly impacted by temperature (Cohen *et al.*, 2012). Distinct pre-veraison water status notably at pea size stage which was not assessed during our study, might help explain the TPI differences between vintages (Zarrouk *et al.*, 2012).

Greater concentrations in amino acids were observed in 2016 during the rainier vintage. In 2015, we can assume that the dry soil conditions experienced in spring and in early summer did not promote the mineralisation of soil organic matter and the assimilation of ions by the root system. Moreover, the cooler nights experienced during maturation in 2016 might also play a role. It has been recently highlighted that cool night index was inversely correlated to several amino acids, suggesting that the synthesis or accumulation of some amino acids might be enhanced by cool temperatures (Gutiérrez-Gamboa *et al.*, 2018). This observation was not confirmed for ammonium and potassium, which are more difficult to interpret due to a significant vintage × treatment interaction.

Concentrations in rotundone up to 69 ng/L were found in wines, which indicates that the molecule is likely to have a large contribution to the sensory profile of Fer wines from the Marcillac area. Rotundone was affected by the vintage, with greater concentrations found in wines from 2015. Higher concentrations would have been expected in 2016, the coolest vintage with the smaller DH25 (Caputi *et al.*, 2011; Zhang *et al.*, 2015). However, Geffroy *et al.* (2014) emphasised that the amount of rainfall in the late berry development stages and the plant water status during maturation were key variables to explain the differences in rotundone between vintages in the southwest of France. Indeed, if 2015 was dryer than 2016 between the 1 January and

31 December period and $\delta^{13}C$ did not allow to discriminate the two vintages, 2015 was wetter over the maturation period. In addition, greater yields and the phenological delay observed in 2016 might have contributed to lowering the sugar concentration in berries at harvest. Even if rotundone appears to be produced in berries (Geffroy *et al.*, 2016; Zhang *et al.*; 2016) and is not impacted by source-sink ratio in conditions favourable to a satisfactory level of maturity (Geffroy *et al.*, 2014), the low level of sugar concentration in 2016 might have negatively impacted the accumulation of rotundone. Actually, as other secondary metabolites, rotundone synthesis must be an energy demanding process that requires carbohydrates. As rotundone is hydrophobic and was measured indirectly in wine, we can also suppose that the difference in ethanol content between the two vintages might have impacted its extraction from the berry pericarp, which is enhanced by fortification and elevated alcohol concentration (Zhang *et al.*, 2017).

IBMP concentrations were below detection level in wines from 2015 which could be a result of the heat wave experienced between berry set and bunch closure in July. Indeed, it has been highlighted that the concentration of IBMP in grapes strongly decreases with temperature (Falcão *et al.*, 2007). In 2016, IBMP concentrations remained below the odour threshold of 15 ng/L established by Roujou de Boubée (2000). With 2 years of IBMP data below the perception threshold of the molecule, it is legitimate to wonder about the sensory impact of IBMP in Fer wines from this region. Research by Poitou *et al.* (2017) after our study was conducted showed that 1,8-cineole, a monoterpene responsible for eucalyptus and mint aroma, could have an additive effect with IBMP and may decrease its perception threshold. As the same authors also highlighted that 1,8-cineole was found at high level in Fer wines from the Marcillac wine region, we can assume that IBMP might play a sensory role in 2016, notably in the control wine whose level of concentration is just below the 15ng/L threshold.

2. Effect of the techniques

The time necessary to remove bunch zone leaves on one side of the row and lateral shoots was estimated at 43 ± 2 hours per hectare and 93 ± 6 hours per hectare, respectively.

Yields at harvest and mass of 200 berries were not impacted by the techniques. Carbohydrate supply at flowering is a key driver of berry set and berry development (Coombe, 1962). For the removal of lateral shoot treatment, this observation was expected because shoots were removed early before bearing two adults leaves and becoming source organs (Kliewer, 1970). For defoliation, this absence of effect is somewhat unexpected, as Kliewer (1970) showed that defoliation after berry set affected berry size. According to this author, the decrease in berry size is more intense when defoliation is performed early just after berry set. Therefore, it appears that the timing of defoliation applied in our experiment 10 days after berry set is appropriate to avoid any crop loss through berry size reduction.

A decrease in yield would have been expected for delayed harvest, a practice which was likely to induce a crop weight loss through water evaporation. Even if the xylem flow is decreased after veraison and the berry volume is less sensitive to soil moisture (Bondada *et al.*, 2005), the transpiration must have been compensated by the large amount of rainfall recorded on the site between the two harvest dates (26 mm in 2015 and 24 mm in 2016). This water accumulation into the berry could explain the decrease of sugar concentration, TPI and anthocyanins, the changes in acidity (i.e. titratable acidity, malic acid and pH) and in potassium (in 2015 only) for this treatment. In addition, the decrease in anthocyanins could be related to the drop of temperature and to the lower level of water deficits experienced by the vine after the first harvest (Roggero *et al.*, 1986; Somers, 1976). The reasons why a decrease in potassium was not observed for the delayed harvest treatment in 2016 remain unclear.

Leaf removal and the removal of lateral shoots had no significant impact on sugar levels which suggests that the vine can compensate the loss in foliar surface induced by these practices. Indeed, most of the researches indicates that compensation can take place through i) a stronger development of lateral shoots for defoliated vines (Kliewer and Fuller, 1973), ii) an increased photosynthesis efficiency of the main leaves when lateral shoots are removed (Candolfi-Vasconcelos *et al.*, 1994), and iii) the translocation of sugars from starch mobilisation in roots and woody fractions to the berries (Zufferey *et al.*, 2012).

TABLE 5. Effect of leaf removal and removal of lateral shoots on bunch surface temperature (mean and standard deviation of 30 observations) and results of the ANOVA.

Bunch surface temperature (°C)						P-value									
Time of measurement (TM)	Side of the row (S)	Block (B)	Control	Leaf removal	Removal of lateral shoots	TM	S	B	T	TM × S	TM × B	TM × T	S × B	S × T	B × T
1200 h	East-facing	1	19.46±1.89	20.54±1.80	19.69±1.47										
		2	18.38±1.16	19.85±1.24	20.35±1.63										
		3	16.09±1.18	16.41±1.39	15.73±1.40										
	West-facing	1	20.31±1.29	20.62±0.91	21.06±1.29										
		2	20.09±0.86	20.36±0.94	20.70±1.22										
		3	18.30±0.79	17.45±0.73	18.25±1.87	<0.001	<0.001	<0.001	0.120	<0.001	<0.001	0.150	<0.001	0.587	0.002
1600 h	East-facing	1	26.15±0.96	25.89±0.69	25.23±0.64										
		2	26.16±0.77	25.79±0.87	25.97±0.92										
		3	25.66±0.64	25.19±0.63	25.69±0.85										
	West-facing	1	30.02±3.09	29.43±3.61	28.19±2.70										
		2	29.83±3.02	31.01±2.59	29.62±2.42										
		3	28.88±2.80	30.18±3.27	31.76±2.77										

Surprisingly, malic acid and in most cases potassium were increased for the defoliated treatment. The increase in berry temperature and the lower foliar density should have enhanced malate degradation and led to a lower potassium content, respectively (Kliewer and Bledsoe, 1987; Smart, 1985; Wolf *et al.*, 1986). The gains observed in our experimental conditions could be attributable to a higher lateral shoot growth. For malic acid, the absence of impact of leaf removal on bunch surface temperature (Table 5) and bunch air temperature between veraison and harvest (i.e. mean and maximal values, cool night index) might also have played a role. This could be explained by the specific features of the experimental site (presence of a hedge of trees on the southern border, steep slope and row orientation) and the development of newly emerged leaves, which must have minimized the penetration of solar radiation within the bunch zone. This likely low impact of solar radiation on berries is also supported by the anthocyanins concentrations that should have increased in both vintages (Tardáguila *et al.*, 2008). However, a gain in anthocyanins was only observed in 2015. During the late 2016 vintage, we can assume that the high solar azimuth angle during the late maturation period limited berry illumination and prevented the pigment synthesis. Trivially, our results also indicated that bunch surface temperature increased between 12:00 and 16:00 h and was higher (in the afternoon only) and more variable as reflected by standard deviation values in the side of the row exposed to direct sun exposure (Table 5) which is relevant with previous researches (Moffat *et al.*, 2013). The reasons why bunch temperature was lower on the east-facing side of the row at 12:00 remain unclear. In most cases, bunch temperature was greater on blocks 1 and 2 that are distant from the hedge of trees.

The removal of lateral shoots had no effect on acidity components and anthocyanins, which also suggests that the technique had a weak impact on bunch microclimate and notably berry temperature as reflected by bunch microclimate measurements. As this technique has been little studied, it remains difficult to generalise these findings due to the specific features of our experimental site (see discussion above).

Leaf nitrogen status (NBI) determined between veraison and harvest did not make it possible to discriminate the treatments. In grapes, the greatest amino acids content was noted for the delayed harvest treatment, which is in accordance with previous research highlighting an increase in amino acids over the maturation period (Kliewer, 1968) and particularly in cool night climatic conditions (Gutiérrez-Gamboa *et al.*, 2018).

In most cases, ammonium content in berries was lowered for leaf removal. More than half of the berries' nitrogen is imported after veraison through the phloem (Keller, 2015). We can assume that the technique induced a decrease in the source-sink ratio which has contributed to lower ammonium in berries. There remains no explanation as to why a loss in amino acids, and losses in amino acids and ammonium were not observed for leaf removal and the removal of lateral shoots treatment, respectively.

Leaf removal as well as the removal of lateral shoots induced an increase in stem water potentials, which could be the consequence of a lesser loss of water through transpiration. These differences in water status were not confirmed through measurements of $\delta^{13}C$.

In 2016, IBMP concentrations were not impacted by the treatment factor at $P<0.05$ but differences were observed at $P=0.06$. The removal of lateral shoots and the delayed harvest treatments showed the lowest IBMP concentrations (-24% and -26% in comparison with the control treatment, respectively). IBMP in wines from the leaf removal treatment did not differ significantly from the control, however a trend of lower levels was found (-12%). These conclusions are consistent with previous findings for delayed harvest (Roujou de Boubée *et al.*, 2000, Ryona *et al.*, 2010) but deserve further discussions for leaf removal and removal of lateral shoots. As in our experimental conditions, berry temperature did not allow to discriminate these two practices, the quantity of light received by the berries is expected to be the main determinant of IBMP in wines. As discussed previously, we can suppose that the development of newly emerged leaves and lateral shoots limited the penetration of light through the canopy and IBMP photodegradation for the defoliated treatment in comparison with the removal of lateral shoots treatment.

For rotundone, the largest gain was observed for the delayed harvest treatment (+121% in 2015 and +120% in 2016) while levels in wines from the leaf removal treatment showed intermediate results in trend (+71% in 2015 and +43% in 2016). Rotundone accumulation during grape maturation is known to increase steadily before plateauing (Geffroy *et al.*, 2014; Zhang *et al.*, 2015). In the southwest of France, this plateau was reached for Duras cultivar 44 days after mid-veraison (Geffroy *et al.*, 2014). In our experimental conditions, the significant increase observed between the two harvest dates indicates that maximum concentrations were not yet reached 54 days after mid-veraison. According to Zhang *et al.* (2015), the rotundone plateau is higher and reached earlier during cooler and wetter growing seasons. We can suppose that the hot climatic conditions observed during maturation in 2015 and to a lesser extent in 2016 might have contributed to delayed rotundone accumulation. Therefore, we can not completely exclude that no gain in rotundone would be observed for the delayed harvest treatment under less hot weather conditions.

The observations on leaf removal somewhat contradict previous research highlighting a decrease in the grape rotundone concentration for defoliated vines (Geffroy *et al.*, 2014) but are in agreement with other findings emphasising an increase (Homich *et al.*, 2017). This non-significant increase cannot be imputable to a stress experienced by physical wounding (Zhang *et al.*, 2016), or to a lesser level of water deficit as the removal of lateral shoots, which induced the same effects, had no impact on rotundone. Recently, Geffroy *et al.* (2019) suggested that light received by the berries could stimulate the production of rotundone. However, if this were to be the mechanism involved a larger gain would have been observed for the removal of lateral shoots treatment, which seems more favourable to light penetration (see discussion above). However, the removal of lateral shoots had no impact on rotundone. Further studies will be necessary to understand the mechanisms involved in rotundone production after defoliation.

When focusing on the ratio of OAV rotundone to OAV IBMP, the three studied techniques made it possible to significantly increase the ratio, with the largest gain observed for delayed harvest.

With only one year of data for IBMP and the ratio of OAV rotundone to OAV IBMP, we cannot exclude the fact that our results should be considered preliminary and that one extra year would be necessary to fully validate our observations. However, our findings appear particularly consistent from one vintage to the other for rotundone (expressed in % of control), which tends to confirm the strong repeatability and robustness of our measurements.

CONCLUSION

Our results indicate that common viticultural practices can be used to modify the composition in rotundone and IBMP of Fer wines. Delayed harvest appears to be the best practice to increase rotundone while decreasing IBMP. In our experimental conditions, this practice had no impact on yields and did not induce any extra costs. Its only drawback was to induce a decrease in anthocyanins. Defoliation, which in 2015 induced a gain in anthocyanins, is also a recommended practice to increase the ratio of OAV of rotundone to OAV of IBMP. In our conditions, 43 hours per hectare were required to perform defoliation, an operation that can be mechanised except for terraces. Despite its relative efficiency, the removal of lateral shoots is not an advisable technique to modify the aroma composition of the wines, as it required more than 90 hours per hectare of manual work.

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Article 7:

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On-vine grape drying combined with irrigation allows to produce red wines with enhanced phenolic and rotundone concentrations

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Résumé : L'absence de contrainte hydrique étant favorable à l'accumulation de la rotundone mais défavorable à celle des polyphénols, il semble délicat d'élaborer des vins riches en rotundone et en composés phénoliques. Afin d'atteindre ce double objectif, le Passerillage Eclaircissage sur Souche (PES), une technique consistant à sectionner 3 semaines avant la récolte la branche à fruit sur une vigne conduite en Guyot, a été testée. Dans un premier temps, nous avons démontré que la technique ne pénalisait pas l'accumulation de la rotundone dans les vins. Dans un second temps, nous avons imaginé et expérimenté avec succès un système associant irrigation puis PES, pour favoriser la synthèse de la rotundone et diminuer l'impact négatif des apports d'eau sur la richesse polyphénolique des vins.

Abstract

Rotundone, the compound responsible for peppery aroma in red wines is positively correlated with the absence of water deficit during ripening which can make it problematic to produce red wines combining both superior concentrations in rotundone and phenolic compounds. To reach this double objective, on-vine drying with cutting of the fruit bearing cane or “Passerillage Éclaircissage sur Souche” (PES), a technique that consists in cutting the cane 2 to 3 weeks prior to harvest on a Guyot-trained vineyard was investigated combined or not with irrigation. We showed that PES had a limited impact on rotundone in wine. As the PES technique leads to an interruption of the sap flow, our results suggest that rotundone is synthesized in the berries and not translocated. A viticultural system combining irrigation and then PES induced significant gains in rotundone, in sugar concentration, in anthocyanins and in Total Phenolic Index in wine, and in skin to juice ratio. Measurements of bunch surface temperature indicate that the enhancement of rotundone production induced by irrigation is likely to be due to a direct rather than to an indirect effect through an increase in leaf area leading to a cooler bunch microclimate.

KeyWords: *rotundone, peppery aroma, phenolic compound, on-vine drying with cutting of the fruit bearing cane, irrigation*

Introduction

Rotundone is the main compound responsible for peppery aroma in red wines which was discovered in 2008 in Australian Shiraz wine and grapes (Wood, et al. 2008). It has been identified in Pinot Noir, Durif, Graciano, Riesling (Herderich, et al. 2012) and more recently in other cultivars such as Duras (Geffroy, et al. 2014), Gamay (Geffroy, et al. 2016), and Malbec, Abouriou (Cullere, et al. 2016) grown in France under cool or Atlantic climate conditions . Differences in rotundone concentrations in wines made from four Duras certified clones suggests that rotundone was involved in the vine's natural response to powdery mildew attacks (Geffroy, et al. 2015). While leaf removal strongly reduces rotundone, irrigation enhances its accumulation in berries (Geffroy, et al. 2014). Other researches have highlighted large spatial variations in berry and wine rotundone concentrations within a single vineyard (Geffroy, et al. 2014,Scarlett, et al. 2014,Zhang, et al. 2015). In most cases, this variability was associated with variation in the land underlying the vineyard and was linked with vine water status. While it was reported that the highest rotundone concentrations were found in wines made from vines experiencing low water deficit within the same vineyard (Geffroy, et al. 2014). Other works showed that rotundone concentration was positively correlated with vineyard water balance in wines produced from 15 vintages and from the same Shiraz vineyard (Zhang, et al. 2015).

Water deficit during ripening is known to reduce berry weight and increase the concentration of anthocyanins and tannins in wines (Bucchetti, et al. 2011,Ojeda, et al. 2002). Indeed, in addition to the indirect increase of phenolic compounds due to berry size reduction, the expression of genes coding for flavonoid and anthocyanin biosynthetic enzymes is strongly enhanced in berries grown under drought conditions (Castellarin, et al. 2007). In most cases and especially in vineyards under oceanic or cool climatic influence experiencing low water deficit, it can be problematic to produce red wines combining both superior concentration in peppery compound and phenolic richness.

On-vine grape drying with cutting of the fruit bearing cane, alsoknown in French as "Passerillage-Éclaircissage sur Souche" (PES) or "double maturation raisonnée" (DMR), is a viticultural technique still little known worldwide. PES consists of cutting the cane 2 to 3 weeks prior to harvest on a Guyot-trained vineyard. Studies carried out since 1992 in Italy (Garofolo, et al. 1993), Croatia (Peršurić, et al. 1998), Switzerland (Rösti, et al. 2011) and France (Serrano, et al. 2007) , showed that this technique implemented on red or white cultivars, caused some reduction in yield and allowed for improving the overall wine quality by increasing sugar and phenolic concentrations in the berries due to dehydration. Corso, et al. (2013) showed that PES induced flavonol and depressed catechin accumulation respectively which led to brighter colored wine with lower astringency. Cutting the cane produces two bunch populations: the first one for which the connection with the trunk is still active and the second for which any connection with the trunk is severed. Seven to eight hours of manual work per hectare were necessary to perform the cutting in the South West of France (Serrano, et al. 2007). Results from the same research work showed that the technique was suitable for mechanical harvesting and that the cutting of fruit cane had no impact on the quantity of waste (i.e. leaves, petioles and stems) or on the harvesting rate. However, PES induced a 3-fold increase of losses due to the fall of grapes and canes onto the ground.

Rotundone was reported in grape leaves and stems (Capone, et al. 2012) and more recently in Shiraz flower caps at pre-veraison (Zhang, et al. 2016). It has been reported that some monoterpene derivatives could be translocated via phloem transportation in other plants (Turgeon and Medville 2004). Therefore, PES that leads to an interruption of the sap flow is susceptible to impact rotundone which could be translocated from vegetative tissues to grape berries.

The aims of this study were to apply PES and to assess its impact on rotundone in wine. A two-year study was conducted in 2013 and in 2014 on Duras, a grape variety grown in the Protected Designation of Origin Gaillac in the south-west of France. Following on from a PES only experiment in 2013, a viticultural system combining five irrigations and PES was implemented in 2014. As irrigation is known to enhance rotundone accumulation (Geffroy, et al. 2014) and, in most cases, increases berry size and dilution of the skin compounds (Ojeda, et al. 2002), this viticultural system was intended to increase rotundone concentrations while mitigating, through PES, the negative effects of irrigation on phenolic concentration of wines.

Material and methods

Experimental site and design

Two separate experiments, the first one concerning only PES (2013 and 2014) and the second one a viticultural system consisting of five irrigations, each one equivalent to 14 mm of rain, and then PES (2014), were established in two separate parts of the same vineyard. The 0.60-ha vineyard, located in the heart of the Gaillac region (lat. 43° 50' 21" N; long. 01° 51' 01" E) and typical of the area with 2.20 m x 1 m vine spacing, was planted in 2001 with Duras (clone designated number 554) grafted on Gravesac rootstock. Orientation of the vine rows was north-east to south-west, and vines were trained with vertical shoot positioning on a single Guyot pruning system. The soil was managed by chemical weed control under the vines and by grass cover in the inter-row area. The experimental unit presented with a slope of 6%. Despite the apparent homogeneity of the plot, trunk circumference (TC) measurements were carried out in order to detect any source of variability. Previously, studies showed a link existed between TC - an index of vine vigor well correlated to apparent electrical conductivity of the soil (ECa) - and rotundone, and that TC could be used to approach rotundone spatial distribution (Geffroy, et al. 2015). Therefore, just before budburst, in April 2013 and in April 2014 for the PES and viticultural system experiments respectively, TC was determined for every vine within each experimental unit as the average value measured at three different heights (just above the graft; 10 cm under the trellis wire; and half way between the two first points of measurement) and confirmed the homogeneity of the plot. Consequently, the two experiments were undertaken using a completely randomized design with four replications. For both experiments, the experimental site was established on four consecutive rows with each row containing two experimental units of 26.4 m^2 composed of 12 continuous vines.

Weather measurements

In 2013 and 2014, rainfall and air temperature (minima, maxima and mean values) were monitored daily by a CimAGRO weather station (Cimel Electronique, Paris, France) placed within 400 m of the experimental site. In order to characterize the two vintages, several indices proposed by Tonietto and Carbonneau (2002) were calculated for the PES experiment: Huglin index or heliothermal index over the period of 1 April to 30 September, the cool night index (FNv-r), the mean air temperature (Tv-r), the maximal air temperature (Txv-r), and the thermal amplitude (Av-r) indices over the veraison–harvest period. Cumulative rainfalls over the whole calendar year, the budburst– veraison, veraison – harvest and cutting – harvest periods were also calculated for the PES experiment.

PES experiment (2013 and 2014)

On 4 out of 8 experimental units, the fruit cane was cut beyond the first growing shoot from the trunk using a manual pruning shear on 26 September in 2013 and on 11 September in 2014, which corresponds to 30 days after mid-veraison. Mid-veraison (50% of colored berries) was determined by collecting 100 berries on the experimental site and by counting the number of colored berries every third day from the end of July to the beginning of August. Harvest took place exactly 48 days after mid-veraison on 14 October in 2013 and on 29 September in 2014. In order to get a better understanding of the impact of the technique on rotundone and classical enological parameters, the bunch population still connected with the trunk (Spur) and the disconnected one (Cane), were monitored separately (Fig. 1) and compared with a control treatment (Control).

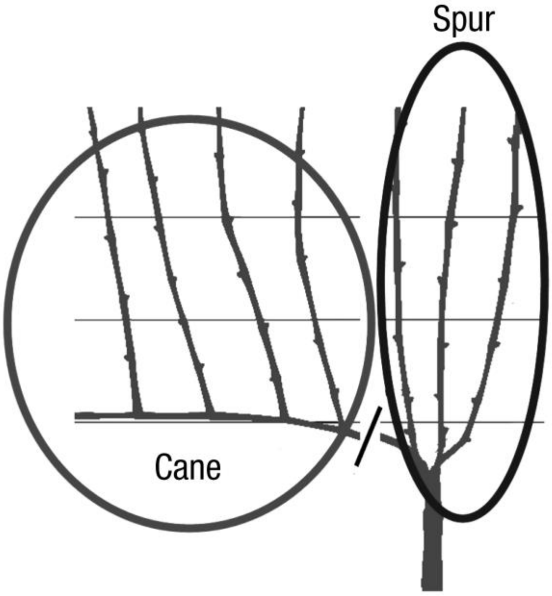

Fig. 1. Principle of on-vine grape drying with the cutting of the fruit bearing cane or "Passerillage Éclaircissage sur Souche" (PES). The spur bunch population is still connected with the trunk while the cane bunch is completely disconnected.

Viticultural system combining irrigation and PES (2014)

The viticultural system combining irrigation and PES (Irrigation + PES) was compared in 2014 with a control treatment (Control). For this system five irrigations, each equivalent to 14 mm of rain, were applied from 30 June (10 days after berry set) at the frequency of one irrigation per week using drip irrigation pipes (6 L/h drippers at 0.5 m spacing). The last irrigation took place 12 days before mid-veraison on July 29. Irrigation volumes were calculated as follows:

Irrigation volume (L/vine plant) = Equivalent of rainfall (mm) x Surface of the plot (m^2) / Number of vine plants of the plot

No differences in the date of mid-veraison determined by counting the number of colored berries from 100 berry samples were observed between the two treatments. Then, the fruit cane was cut beyond the first growing shoot from the trunk using a manual pruning shear on September 9 which corresponds to 30 days after mid-veraison, and harvest took place 18 days later on 27 September. In order to get into industrial conditions for which grapes are usually mechanically harvested and cannot be sorted according to bunch population, the two bunch populations were monitored together.

Grape sampling, analysis of conventional enological parameters and $\delta^{13}C$ measures

Just before cutting the canes for each of the two experiments, eight grape samples were collected (one per experimental unit). At harvest, 12 and 8 grape samples were collected for the PES experiment (four per bunch population and four for the control) and for the viticultural system experiment (four for the control and four for the Irrigation + PES treatment) respectively. Each sample consisted of 216 healthy berries from both sides of the row and several parts of the bunch (nine berries per vine on each side of the row). Prior to start the analysis the weight of 200 berries was measured. For the samples collected at harvest on the viticultural system experiment, withered berries belonging to the *cane* population that could be easily distinguished visually were also weighed separately in our laboratory. Then the berries were crushed gently, and the musts obtained were centrifuged (14 000 × *g* for 6 min) to enable classical laboratory and $\delta^{13}C$ analyses. Sugar concentration (°Brix) was determined with a digital hand-held Pocket refractometer PAL (Atago, Japan). Titratable acidity was measured according to OIV method (2009) and pH was measured with a HI 3221 pH meter (Hana Instruments, France). A Konelab Arena 20 sequential analyzer (Thermo Electron Corporation, USA) associated with enzyme kits provided by several suppliers was used to determine amino acids, ammonium, gluconic acid in 2013 only (Megazyme, Ireland) and malic acid (Thermo Fisher Scientific, USA). Potassium determination was by flame photometry (Bio Arrow, France) according to OIV method (2009) and tartaric acid determination by colorimetric titration (Hill and Caputi 1970). The remaining berries were crushed for two minutes at low speed (600 rpm) using a food blender (Moulinex, Faciclic, France) to perform phenolic analyses. Anthocyanins and Total Phenolic Index (TPI) were quantified in grapes according to the method described by Cayla, et al. (2002) which can be summarized here briefly. 50 g of the crushed berries were macerated for 1 hour in a medium containing 15 mL of ethanol (95%) and 85 mL of HCl (37%). Samples were stirred every fifteen minutes and after centrifugation (14 000 × *g* for 6 min), anthocyanins and TPI were quantified the same way as in wine according to (Ribéreau-Gayon and Stonestreet 1965) and (Ribéreau-Gayon 1970) respectively using an Evolution 100 spectrophotometer (Thermo Electron Corporation, USA). This method shows good reproducibility (average coefficient of variation of 5%) and correlations (R^2>0.95) with other methods for phenolic determination in grapes. All determinations were carried out in duplicate. For the control treatment of the PES experiment, $\delta^{13}C$ – a proxy for vine water status (Van Leeuwen, et al. 2009) - was determined at harvest on each experimental unit according to the protocol described by Geffroy, et al. (2014).

Microvinification and rotundone analysis.

Because of quarantine requirements and logistical issues with shipping frozen grapes from France to Australia, rotundone was analyzed indirectly in wines fermented under microvinification conditions. 12 grape samples of 800 g of healthy berries, from both sides of the row and several parts of the bunch, were collected at harvest for the PES experiment (four per bunch population and four for the control) and eight samples for the viticultural system. Mass proportions of Cane berries were determined the same way as described previously. Microscale fermentations in a 1 L Erlenmeyer flask were carried out according to the protocol proposed Geffroy, et al. (2014). After 8 days of fermentation, the amount of wine and of skins was measured to determine skin-to-juice ratio at pressing (%). Wines were centrifuged (14 000 × *g* for 6 min), analyzed for TPI and anthocyanins according to Ribéreau-Gayon and Stonestreet (1965) and Ribéreau-Gayon (1970), and received a sulphite addition of 80 mg/l before to be bottled and stored at 4°C or below until rotundone analysis. Rotundone in wine was determined by solid phase microextraction-multidimensional gas chromatography-mass spectrometry according to the protocol described by Geffroy, et al. (2014). For the rotundone, reproducibility tests showed an average coefficient of variation of 5% across the microfermentors (Geffroy, et al. 2014). Rotundone is a hydrophobic compound being mainly extracted from the berries between days 2 and 5 of fermentation under the effect of ethanol (Siebert and Solomon 2010). The impact of differences in sugar content between the samples leading to distinct ethanol in the resulting wines on rotundone extraction must remain negligible as the impact of ethanol on the extraction of hydrophobic compounds from the berry skin such as proanthocyanidins didn't exceed a few percent on mature or almost mature grapes (Canals, et al. 2005). As all the experimental wines were made under the same conditions with good reproducibility of the microscale ferments, the concentration of rotundone found in the wines was considered to reflect the rotundone concentration in the corresponding berry samples. The analyses were performed in two different years but during the same period of each year to reduce potential variations associated with different post-bottling times.

Vine measures

For the two experiments, yield at harvest (kg/vine) was monitored for each vine by weighing individually crop load with a Precia Molen C20 K balance (Precia SA, Privas, France). The two bunch populations were always weighed separately. For the viticultural system experiment, these measurements were conducted before pooling together the two bunch populations to verify the representativeness of the sampling and to ensure the proportion of Spur and Cane berries was respected. For the viticultural system experiment, water status of the vine was measured every two weeks, from the end of June to the beginning of September, on the two studied treatments by stem water potential (Ψstem) according to the protocol defined by (Choné, et al. 2001). For each experimental unit, measurements were taken from 3 of the 12 vines and from two mature exposed leaves per vine. The three vines were marked, and stem water potential was always measured on these selected plants. On cloudless days, leaf blades were initially enclosed in plastic bags between 11:00 and 12:00 h. Measurements were made between 14:00 and 15:00 h. Severe bunch rot (*Botrytis cinerea*) and acid rot (*Drosophila melanogaster* and/or *Drosophila suzukii*) damages, whose development occurred during the cutting to harvest period, were recorded on grapes in 2013 and 2014 respectively. Frequency and severity was assessed at harvest by visually estimating the percentage of the bunch area colonized by the pathogen or damaged by the insect on 100 grape bunches (50 per each side of the vine) within each experimental unit. For the PES experiment, the two bunch populations were monitored separately. No damages due to bunch rot in 2014 and acid rot in 2013 were observed on the experimental plots.

Bunch surface temperature

For the viticultural system experiment in order to investigate whether the enhancement of rotundone production induced by irrigation previously described by (Geffroy, et al. 2014) was due to a direct or indirect effect through an increase in leaf area (Ginestar *et al.*, 1998) leading to a cooler bunch microclimate, bunch surface temperature was measured halfway between the two end points of the bunch using a non-contact Raynger MX infrared thermometer (Raytek, USA). Measurements were made on 50 bunches for each experimental unit and on each side of the vine at 12:00 and 16:00 h on September 3, a cloudless and windless day corresponding to halfway between mid-veraison and harvest, and 6 days before cutting the canes. The measurements in the experimental plot were completed in less than 30 minutes for each time of measurement. Bunch surface temperature was first to be assessed for all the experimental units on the east facing side and then on the west facing side of the row. Two temperature loggers (EBI 20-T1, Ebro Electronic, Germany) attached to bunch pedicels on both sides of the canopy for the control treatment and shaded by bunch zone leaves to minimize direct solar exposure showed that the variation of temperature in the bunch area over the measurement period for each side of the row was limited to ± 0.3°C. Solar radiation data obtained from HelioClim-3, a satellite-based surface solar irradiation database (Blanc, et al. 2011,Eissa, et al. 2015), showed that the radiation environment was stable (with variation in irradiation less than 5%) through the 15-minute period required to collect data from one side of the row.

Statistical analysis

Statistical analyses were conducted with Xlstat software (Addinsoft, Paris, France). All the analytical data in 2013 and 2014 from the PES experiment were first subjected to a two-way analysis of variance (ANOVA) treatment (vintage × treatment) with first-order interaction.

Because vintage × treatment interactions were almost always significant (results not shown), data were next subjected for each year to a one-way ANOVA (treatment). For the viticultural system experiment, data were analyzed by a one-way ANOVA (treatment) with the exception of bunch surface temperature data that were subjected to a three-way ANOVA treatment (treatment × side of the row × time of measurement) with first-order interaction. Fisher's least significant difference test was used as a post-hoc comparison of means at $P < 0.05$.

Results and discussion

Effect of vintage on fruit and rotundone

The 2013 vintage was characterized by a cold spring, regular and large rainfall events over the vine vegetative cycle which induced a delay of 3 weeks at harvest and ripening difficulties. In 2014, winter was rainy and warm; summer was extremely rainy with 160 mm of rain recorded between mid-July and the end of August and conditions during ripening were dry and hot. Climatic indices and cumulative rainfalls calculated for the two vintages of study are presented in Table 1. The 2013 vintage was cooler, less rainy than 2014 whose rainfalls events mostly occurred as summer storms and, the two vintages were characterized by comparable $\delta^{13}C$ values and consequently by a similar level of water deficit.

As reflected by the control data just before cutting the canes (Table 2) and as a consequence of lower grape yields (Bravdo, et al. 1985), the 2013 vintage showed a higher level of ripeness at [mid-veraison + 30 days], i.e. higher sugar concentration, pH, anthocyanins, TPI, and lower titratable acidity than 2014. Indeed, the summer rainfall events which occurred during the berry growth period in 2014 induced a large increase in berry mass associated with a decrease in skin to juice ratio which is in agreement with observations by Stevens, et al. (1995) that showed a strong correlation between berry growth and water applied. At harvest, 18 days after performing the cutting, surprisingly the 2014 vintage showed a lower level of titratable acidity, and higher pH, anthocyanins and TPI than found in 2013. In 2014 just before harvest, climatic conditions were dry and hot which enhanced ripeness while in 2013 during the same term, the sunlight period and temperature were lower and less favorable to ripening due to the three-week delay in phenology.

Table 1. Characterization of the 2013 and 2014 vintages by several climatic indexes, cumulative rainfalls and δ^{13}C at harvest.

Vintage	Huglin index (IH)	Cool night index (FNv-r)	Mean air temp (Tv-r)	Maximal air temp (Txv-r)	Thermal amplitude (Av-r)	Cumulative rainfalls (mm)				Water stress δ^{13}C[a]
						01/01 - 1/12	Budburst - veraison	Veraison - harvest	Cutting - harvest	
2013	1901	12.1	17.2	23.6	11.5	700	311	111	43.5	-27.6 no water deficit[b]
2014	2019	13.2	18.9	25.8	12.6	782	331	58	13	-27.8 no water deficit

[a] Values measured on the control treatment of the PES experiment (mean of four replicates).
[b] Thresholds defined by van Leeuwen et al. (2009).

As for rotundone in wine (Fig. 2A), higher concentrations were observed in 2013, the cooler vintage. In the south west of France, Geffroy, et al. (2014) showed that vine water status over the veraison–harvest period was identified as a key variable to explain differences in rotundone between vintages. As 2013 and 2014 are characterized by a similar level of water deficit, temperature appears to be the main determinant of rotundone in grape berries to explain the differences observed between the two vintages. Indeed, in 2014 maximal air temperature during ripening was 25.8 °C compared to 23.6°C in 2013, which is consistent with modeling showing that berry temperature exceeding 25 °C in fruit zone from veraison to harvest negatively affected the rotundone concentration in Shiraz (Zhang, et al. 2015).

As berries infected by powdery mildew can induce plant defence mechanisms leading to synthesis of rotundone in the neighboring healthy berries with concentrations in experimental wines reaching 390 ng/L (Geffroy et al., 2015b), differences in severity of bunch and acid rots might also play a role in explaining differences in rotundone between seasons.

Table 2. Effect of on-vine drying on classical laboratory analysis of the grape at harvest, TPI and anthocyanins in wine, yield components, acid rot and bunch rot in 2013 and 2014. Classical analytical characteristics of the grape before cutting the canes are also presented. Means followed by the same letter within a row for each period of sampling are not significantly different least significant difference $P \leq 0.05$.

	Before cutting the canes			Harvest			
Parameter	**Control**	**Spur+Cane**	***P*-**	**Control**	**Spur**	**Cane**	***P*-value**
2013							
Mass of 200 berries (g)	318 ± 18 a	325 ± 6 a	0.226	333 ± 2 b	351 ± 6 a	294 ± 8 c	<0.0001
Sugar concentration (°Brix)	20.4 ± 0.2 a	20.4 ± 0.3 a	0.340	21.8 ± 0.3 c	22.1 ± 0.2 b	22.4 ± 0.2 a	<0.0001
Titratable acidity (g/L tartaric	10.93 ± 0.10	11.04 ± 0.19	0.470	9.39 ± 0.21 b	9.45 ± 0.15 b	10.98 ± 0.39	<0.0001
pH	2.91 ± 0.01 a	2.90 ± 0.01 a	0.741	3.01 ± 0.01 a	2.98 ± 0.01 a	2.94 ± 0.02 b	0.002
Tartaric acid (g/L)	4.43 ± 0.09 a	4.39 ±0.11 a	0.458	3.64 ± 0.08 a	3.57 ± 0.14 a	3.49 ± 0.22 a	0.414
Malic acid (g/L)	6.86 ± 0.22 a	6.87 ± 0.34 a	0.684	6.10 ± 0.10 b	6.22 ± 0.09 b	7.32 ± 0.24 a	<0.0001
Potassium (g/L)	1.08 ± 0.10 a	1.01 ± 0.04 a	0.339	1.66 ± 0.01 b	1.79 ± 0.03 a	1.84 ± 0.09 a	0.033
Amino acids (mg/L)	103 ± 5 a	104 ± 2 a	0.616	103 ± 5 a	102 ± 8 a	102 ± 4 a	0.697
NH_4^+ (mg/L)	52.7 ± 3.4 a	52.3 ± 3.9 a	0.277	39.0 ± 4.0 a	34.9 ± 5.9 a	45.5 ± 7.4 a	0.483
TPI grape	84.6 ± 5.7 a	82.2 ± 5.1 a	0.780	62.2 ± 2.7 b	67.7 ± 4.4 a	62.5 ± 5.4 b	0.002
Anthocyanins grape (mg/kg)	1013 ± 65 a	984 ± 49 a	0.697	614 ± 31 b	662 ± 28 a	607 ± 38 b	0.007
TPI wine	-[a]	-		28.7 ± 1.1 b	34.2 ± 0.8 a	29.5 ± 1.0 b	0.001
Anthocyanins wine (mg/L)	-	-		144 ± 7 b	222 ± 20 a	167 ± 3 b	0.001
Skin to juice ratio (%)	-	-		21.4 ± 1.6 b	21.2 ± 1.3 b	24.3 ± 0.09 a	<0.0001
Number of clusters per vine	-	-		19.3 ± 5.5 a	4.3 ± 2.4 c	13.9 ± 4.0 b	<0.0001
Grape yield (kg/vine)	-	-		2.22 ± 0.93	0.28 ± 0.25 c	1.54 ± 0.54	<0.0001
Frequency of bunch rot (%)	-	-		92.6 ± 12.8	93.0 ± 6.1 a	97.0 ± 5.2 a	0.675
Severity of bunch rot (%)	-	-		37.9 ± 22.0	44.7 ± 30.7 a	44.7 ± 26.4	0.554
Gluconic acid (mg/L)	-	-		182 ± 27 a	178 ± 28 a	169 ± 17 a	0.791
2014							
Mass of 200 berries (g)	445 ± 6 a	442 ± 17 a	0.338	461 ± 5 a	489 ± 29 a	322 ± 9 b	<0.0001
Sugar concentration (°Brix)	17.8 ± 0.2 a	17.8 ± 0.3 a	0.356	20.6 ± 0.4 b	21.2 ± 0.3 ab	21.8 ± 0.4 a	0.019
Titratable acidity (g/L tartaric	13.39 ± 0.19	13.20 ± 0.49	0.602	8.79 ± 0.16 b	9.28 ± 0.51 b	11.61 ± 0.69	<0.0001
pH	2.77 ± 0.01 a	2.75 ± 0.02 a	0.127	3.08 ± 0.03 a	3.05 ± 0.03 a	2.98 ± 0.03 b	0.006
Tartaric acid (g/L)	1.96 ± 0.04 a	1.91 ± 0.07 a	0.240	3.27 ± 0.12 a	3.20 ± 0.24 a	2.85 ± 0.12 b	0.014
Malic acid (g/L)	9.81 ± 0.21 a	9.39 ± 0.71 a	0.300	6.00 ± 0.04 b	6.62 ± 0.24 b	8.48 ± 0.65 a	0.001
Potassium (g/L)	1.15 ± 0.04 a	1.13 ± 0.05 a	0.465	1.33 ± 0.03 b	1.45 ± 0.07 a	1.46 ± 0.02 a	0.003
Amino acids (mg/L)	122 ± 13 a	113 ± 6 a	0.251	124 ± 11 a	118 ± 9 a	122 ± 15 a	0.786
NH_4^+ (mg/L)	72.1 ± 10.6 a	68.1 ± 5.6 a	0.286	66.2 ± 9.2 a	63.6 ± 11.0 a	73.0 ± 2.4 a	0.312
TPI grape	79.6 ± 6.7 a	76.6 ± 6.7 a	0.424	64.1 ± 3.2 c	75.0 ± 7.0 b	95.4 ± 5.9 a	<0.0001
Anthocyanins grape (mg/kg)	860 ± 53 a	857 ± 87 a	0.961	818 ± 33 b	899 ± 124 b	1220 ± 80 a	<0.0001
TPI wine	-	-		47.4 ± 2.4 b	47.2 ± 2.4 b	61.4 ± 8.1 a	<0.0001
Anthocyanins wine (mg/L)	-	-		676 ± 24 b	708 ± 46 b	856 ± 105 a	0.004
Skin to juice ratio (%)	-	-		20.0 ± 1.2 b	20.9 ± 0.7 b	25.6 ± 0.4 a	<0.0001
Number of clusters per vine	-	-		15.8 ± 4.0 a	4.2 ± 1.83 c	11.6 ± 3.9 b	<0.0001
Grape yield (kg/vine)	-	-		3.17 ± 0.90 a	0.75 ± 0.45 c	1.75 ± 0.74 b	<0.0001
Frequency of acid rot (%)	-	-		68.5 ± 11.0 a	61.8 ± 10.1 a	73.6 ± 12.4 a	0.372
Severity of acid rot (%)	-	-		8.28 ± 11.6 a	8.44 ± 10.0 a	7.91 ± 9.85 a	0.911

[a] not determined.

Concentrations of 179 ng/L and 89 ng/L were found in 2013 and 2014 respectively in wines prepared by microvinification technique from berries collected from a neighboring healthy Duras vineyard (lat. 43° 50' 36'' N; long. 01° 51' 20'' E) located less than 700 meters away from this experimental site (O. Geffroy, unpublished data, 2015). The absence of bunch and acid rots was confirmed visually and by determinations of gluconic acid for *B. cinerea*. This plot presented the same soil characteristics, the same training system and was planted only two years earlier in 1999 with the same clone and rootstock. It was subjected to similar environmental conditions, to similar viticultural practices and was harvested at a comparable time point after mid-veraison. Moreover, as reflected by $\delta^{13}C$ values measured on grapes at harvest (-27.3 in 2013 and -28.2 in 2014), this vineyard experienced a similar level of water deficit as for our experimental plot (Table 1). As concentrations found in our experimental wines (172 ng/L ± 13 in 2013 and 75 ng/L ± 12 in 2014) are in the same range than these quantifications carried out in wine made from healthy berries, our results suggest that bunch and acid rots don't induce the same natural response as powdery mildew, leading to the production of rotundone. This is particularly surprising for acid rot (*D. melanogaster* and/or *D. suzukii* damages) as with several other sesquiterpenes, rotundone could be involved in the natural defence mechanisms of the vine, especially through the mevalonate/jasmonic acid pathway in response to insect attacks (D'Onofrio *et al.*, 2009).

For the PES experiment, analyses carried out on grapes in 2013 and in 2014 before cutting the canes (Table 2) showed no differences between the two treatments which confirms the homogeneity of the experimental plot. Despite severe bunch rot damage in 2013 that should have brought some perturbation in the data set for most of the measured parameters including sugar concentration (Ky, et al. 2012), small variabilities as reflected by values of standard deviation are observed between the replicates. No significant differences in frequency and severity of acid rot were observed between the treatments. The decrease in severity of bunch rot previously reported with this technique (Serrano, et al. 2007) was unable to be confirmed under our experimental conditions by visual observations (Table 2). Gluconic acid, a secondary metabolite of *B. cinerea*, can also be used as an indicator of the fungal attack and its content is correlated with the percentage of rotten grapes visually estimated (Bocquet, et al. 1995). Even though the sampling was limited to berries with a healthy appearance, significant concentrations of gluconic acid were found in the samples. The absence of differences between the treatments for this parameter indicates that no sampling bias due to *B. cinerea* has been introduced in the data set.

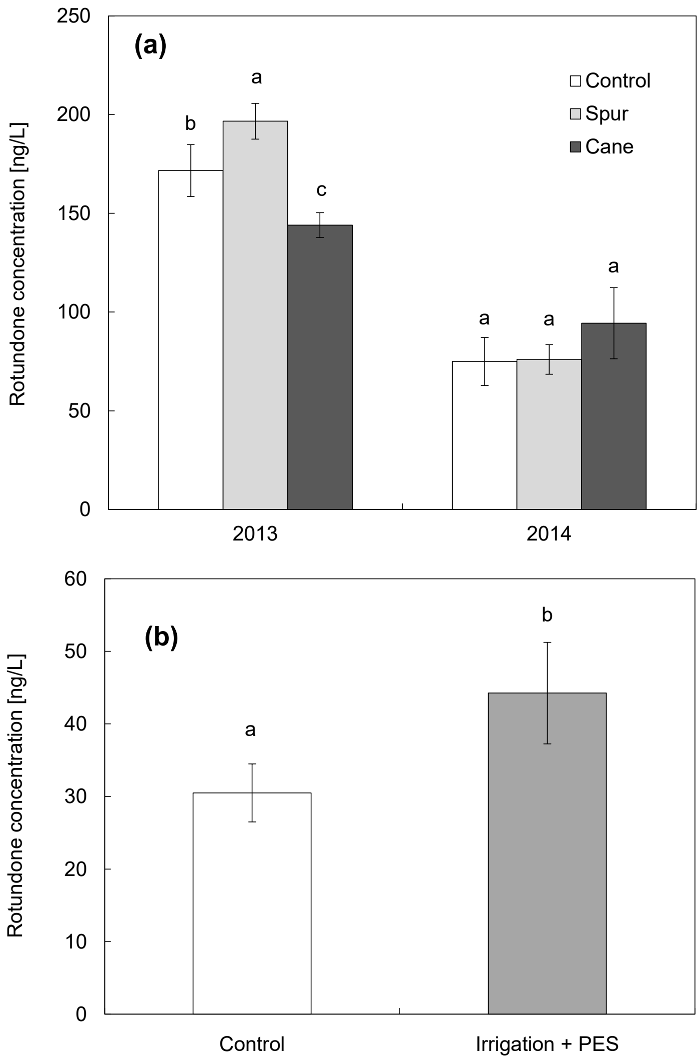

Fig. 2. Impact of (a) on-vine grape drying with cutting of the fruit bearing cane (PES) and (b) a viticultural system combining five irrigations of 14 mm first and then PES on rotundone concentrations in wines. Different letters indicate means significantly different least significant difference $P < 0.05$.

Effect of on-vine drying with cutting of the fruit bearing cane

From the cutting of the canes time point to harvest, a large decrease in phenolic compounds in grape berries was observed in 2013 which must be the consequence of the oxidation of these compounds by laccase from *B. cinerea* (Ky, et al. 2012) whose development occurred during the cutting – harvest period. The same difference was also observed between the determinations made in grape and in wine. From a general point of view, results obtained for the PES experiment are consistent with research on the PES technique conducted in Switzerland between 2002 and 2008 on several cultivars (Rösti, et al. 2011). For the Cane population, the berry mass was reduced (-11.7% in 2013 and -30.2% in 2014) and the skin to juice ratio was improved (+13.6 % in 2013 and +28.0% in 2014) in comparison with the control treatment. These observations showed a larger variation in berry mass and skin to juice ratio for the 2014 samples and suggest a higher concentration by dehydration in 2014. The 2014 vintage was characterized by windier conditions in the last stage of ripening due to the Autan, a föhn-type wind blowing across the south west area. Together with dryer climatic conditions after the cutting (Table 1), it must have contributed to superior grape water losses through evaporation.

For Cane, the dehydration induced an increase in sugar, potassium, malic acid and titratable acidity associated with a decrease in pH. In 2014, the tartaric acid content was significantly reduced for this bunch population which might be the consequence of i) the higher levels of potassium that modified the acid-base balance, provoking a higher tartrate precipitation (Mpelasoka, et al. 2003) and, ii) the absence of translocation of tartaric acid synthetized in the young leaves (Mpelasoka, et al. 2003) after the cutting due to the interruption of the sap flow. Surprisingly, anthocyanins and TPI at harvest were only improved for Cane in 2014. The Spur bunch population showed characteristics similar to the control in 2014, while in 2013 increases were observed in berry mass, sugar concentration and potassium, as well as in anthocyanins and TPI. These increases might reflect compensation mechanisms due to the lesser dilution in the berries by the water absorbed by the vine for berry mass, and of photoassimilates and potassium translocated from the leaves for the other parameters. During this late vintage with ripening difficulties, the cutting of the fruit bearing cane enhanced the ripening of the Spur population. The differences between the two vintages are consistent with the several vintage x treatment interactions observed for the two-way ANOVA treatment first performed on the PES data (see M&M) and are also relevant with previous research by Rösti, et al. (2011). This work showed the dependence of the results when implementing PES in-vineyard with respect to weather conditions.

The same observations can be made for the pepper aroma compound, rotundone, measured in experimental wines. In 2013, concentrations in the Spur treatment for which the ripening was prolonged were higher but for Cane the concentrations were lower (Fig. 2A). Even if significant differences were noticed, they remain weak in terms of intensity. In 2014, the technique didn't impact significantly the rotundone concentration and more variability was observed within the data set. The cutting of the fruit bearing cane seems to have slowed down the accumulation of rotundone which can be formed by direct synthesis from α-Guaiene (Takase, et al. 2016) or accelerated its degradation in Cane in 2013 without this being observed in 2014. Mechanisms involved appear to be complex and rotundone concentration in experimental wines is the resultant of both accumulation/degradation in the berries and extraction during alcoholic fermentation. On the one hand, the foliage desiccation induced by PES 2 to 3 days after the cutting of the canes might have contributed to lower rotundone in wines by increasing bunch surface temperature through a higher solar exposure (Zhang, et al. 2015). On the other hand, the dehydration which provoked an increase in skin to juice ratio might have played a role in increasing rotundone, an aroma compound located in the grape berry exocarp (Siebert and Solomon 2010). Recently, accumulation kinetics of rotundone in berries were modelled through the ripening period according to phenological and thermal times (Zhang, et al. 2015). Rotundone concentrations were shown to reach a plateau which was not achieved for Duras grown in the South West of France before 44 days after mid-veraison (Geffroy, et al. 2014). Therefore, the cutting of the canes 30 days after mid-veraison, is likely to have been performed before reaching the plateau. As the PES technique had a limited impact on rotundone accumulation and induced an interruption of the sap flow, our results suggest that rotundone is synthesized in the berries, and not in the leaves and then translocated to the fruits. This outcome is relevant with previous observations reporting the likely absence of source-sink relationship between leaves and grape berries as grape thinning had no impact on rotundone in wine (Geffroy, et al. 2014).

Effect of a viticultural system combining irrigation first and then PES

The level of homogeneity of the experimental plot was strengthened by the stem water potential measurements performed just before to start the irrigation that didn't show any differences between the two treatments (Fig. 3). From the 4th Ψstem measurements (i.e. the third irrigation), a significant difference in water status was observed between the two treatments. Despite the wet climatic conditions for summer 2014, a 0.10 to 0.15 MPa difference in stem water potential was maintained until the last measurement in early September. As reflected by the analyses performed before the cutting (Table 3), the five irrigations equivalent in total to 70 mm of rain, had a low impact on conventional enological parameters. The sugar concentration was slightly reduced which deserves further comments. As Bravdo, et al. (1985) observed that irrigation induced a reduction in sugar concentration when the dilution caused by the berry growth was faster than the increase in sugar transport into the berry, the irrigated treatment in our experiment appeared to be more favorable to dilution even though the increase in mass of 200 berries was not significant. Despite the absence of differences between the two treatments in the date of mid-veraison, irrigation may also have contributed to slightly enhance shoot growth and delay the onset of ripening (Kliewer, et al. 1983) in our oceanic climatic influence where vines experience low water deficit.

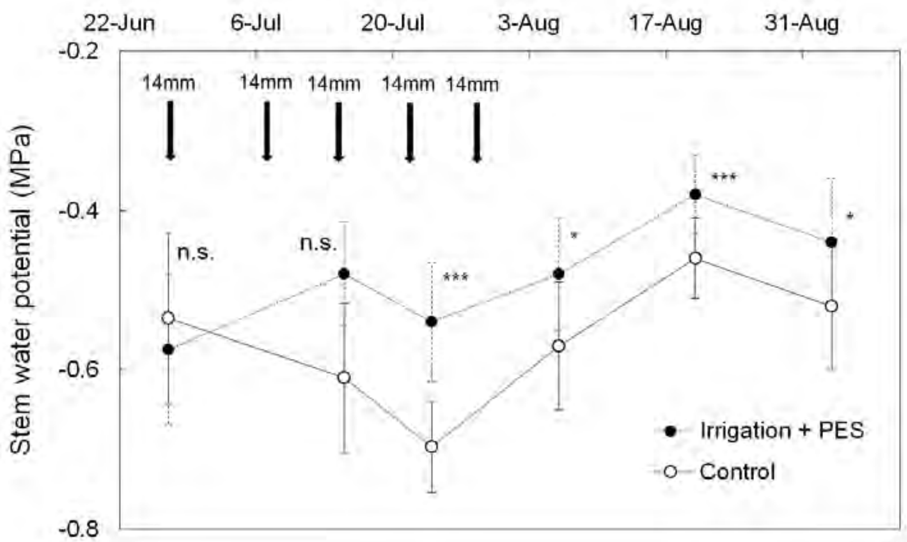

Fig.3. Stem water potential for two water status treatments investigated on the viticultural system experiment in 2014 before to perform the cutting. Irrigations are indicated by black arrows. *: P <0.05; **: P <0.01; *** P <0.001; n.s., not significant

Mass proportions of Cane berries determined in the laboratory were 80.5 % ± 2.2 and 81.4% ± 2.5 for the samples collected at harvest for conventional enological and rotundone analyses respectively. In the field, the same ratio calculated through the measurement of crop load was 82.1% ± 14.3 which confirms the representativeness of the sampling method. A larger variability was observed in the field for which repeated measurements were carried out per replicate and standard deviations refer both to variability within one replicate and to inter-replicate variability. The loss in berry mass and the gain in skin to juice ratio were more marked for the viticultural system in comparison with the PES experiment.

Table 3. Effect of a viticultural system combining irrigation and on-vine grape drying on classical laboratory analysis of the grape at harvest, TPI and anthocyanins in wine, yield components, and acid rot in 2014. Classical analytical characteristics of the grape before cutting the canes are also presented. Means followed by the same letter within a row for each period of sampling are not significantly different least significant difference $P \leq 0.05$.

Parameter	Before cutting the canes			Harvest		
	Control	Irrigation + PES	*P*-value	Control	Irrigation + PES	*P*-value
Mass of 200 berries (g)	430 ± 7 a	436 ± 9 a	0.298	438 ± 11 a	350 ± 12 b	<0.0001
Sugar concentration (°Brix)	20.9 ± 0.2 a	20.3 ± 0.2 b	0.040	22.4 ± 0.3 b	25.4 ± 0.8 a	<0.0001
Titratable acidity (g/L tartaric acid)	11.94 ± 0.22 a	12.24 ± 0.31	0.165	8.10 ± 0.24	9.67 ± 0.39 a	<0.0001
pH	2.86 ± 0.04 a	2.84 ± 0.04 a	0.483	3.15 ± 0.04	3.13 ± 0.02 a	0.262
Tartaric acid (g/L)	2.43 ± 0.08 a	2.38 ± 0.06 a	0.317	3.08 ± 0.12	2.85 ± 0.12 a	0.131
Malic acid (g/L)	8.18 ± 0.22 a	8.22 ± 0.55 a	0.899	5.75 ± 0.10	7.80 ± 0.49 a	<0.0001
Potassium (g/L)	1.37 ± 0.08 a	1.24 ± 0.08 a	0.058	1.51 ± 0.06	1.60 ± 0.10 a	0.148
Amino acids (mg/l)	103 ± 11 a	100 ± 7 a	0.730	134 ± 9 a	122 ± 9 a	0.139
NH_4^+ (mg/l)	50.8 ± 9.5 a	51.9 ± 7.5 a	0.865	61.6 ± 2.4 a	62.7 ± 7.5 a	0.788
TPI grape	94.5 ± 5.8 a	94.6 ± 2.7 a	0.988	81.9 ± 8.3 a	93.1 ± 12.0 a	0.176
Anthocyanins grape (mg/kg)	1077 ± 19 a	1077 ± 39 a	0.994	957 ± 87 a	1001 ± 87 a	0.606
TPI wine	-[a]	-		69.6 ± 2.1 b	85.0 ± 2.1 a	<0.0001
Anthocyanins wine (mg/L)	-	-		1061 ± 53 b	1199 ± 20 a	0.003
Skin to juice ratio (%)	-	-		20.8 ± 1.4 b	27.9 ± 1.4 a	<0.0001
Number of clusters per vine	-	-		14.3 ± 4.2 a	13.6 ± 4.7 a	0.523
Grape yield (kg/vine)	-	-		2.12 ± 0.92	1.78 ± 0.65 b	0.049
Frequency of acid rot (%)	-	-		66.1 ± 4.5 a	57.1 ± 4.5 a	0.218
Severity of acid rot (%)	-	-		8.06 ± 11.1	4.92 ± 5.29 b	<0.0001

[a] not determined.

This suggests a higher concentration for the viticultural system experiment established on the top part of the plot, less vigorous and more exposed to the Autan wind. The studied system induced significant gains in sugar concentration (+3.0 °Brix), in anthocyanins (+138 mg/L) and in TPI (+15.4 units) in wine, and in skin to juice ratio (+7.1%). While the technique had a significant impact on anthocyanins and TPI measured in wines, no differences were observed for these two parameters measured on grapes. This suggests that the analytical method for phenolic determination in grapes didn't allow a complete extraction of the phenolic compounds. Corso, et al. (2013) reported previously that PES induced a modification of the phenolic composition and this change is likely to have led to a lesser extractability under the analytical conditions without this being observed during the 8 days of maceration in 1 L Erlenmeyer flask.

A reduction in severity of acid rot was also observed for *Irrigation + PES* which might be the consequence of a lesser palatability of withered berries and/or difficulties for the insect to perforate the thicker skin with its ovipositor (Lee, et al. 2011).

In comparison with the control, the rotundone concentration (Fig. 2B) was significantly increased by 45.1% for the *Irrigation + PES* treatment in accordance with previous research works (Geffroy, et al. 2014). In the same viticultural context and on the same cultivar, four irrigations each equivalent to 10 mm of rain induced an increase in rotundone ranging from 29% to 38%. Bunch surface temperature measurements (Table 4) showed no significant differences between the two treatments. This suggest that under our experimental conditions (oceanic climatic influence with no or very low water deficit), the increase in rotundone induced by irrigation is more likely to be due to a direct effect rather than an indirect effect due to an increase in leaf area (Ginestar, et al. 1998) leading to a cooler bunch microclimate. The application of regulated deficit irrigation (RDI) is known to modify the hormonal status of berries (Niculcea, et al. 2014). In comparison with a water deficit treatment applied from fruit set to onset of veraison, a full irrigation treatment induced significant gains at veraison in indole-3-acetic acid (IAA), jasmonic acid (JA) and salicylic acid (SA), associated with a decrease in abscisic acid (ABA). These changes in berry phytohormonal content especially the increase in JA concentration for the argument previously discussed (D'Onofrio, et al. 2009) might have contributed to enhance rotundone production in berries from veraison. In a previous study, the foliar application of exogenous jasmonic acid was investigated 20 days after mid-veraison

(Geffroy, et al. 2014). No significant impact was observed on rotundone in experimental wines which is not completely in contradiction with our hypothesis. According to this author, it was difficult to draw conclusions about the role of jasmonic acid as the efficacy of spraying elicitors was strongly dependent on the conditions of the application and especially the penetration of the canopy. Trivially, our results also showed that bunch temperature increased between 12:00 and 16:00 h and was higher and more variable as reflected by standard deviation values in the side of the row exposed to direct sun exposure which is relevant with previous observations made in South Africa on Shiraz grapes (Moffat, et al. 2013).

Table 4. Effect of five irrigations of 14 mm on bunch surface temperature in 2014 and results of three-way analysis of variance. Measurements were made on the viticultural system experiment 6 days before cutting the canes on a cloudless and windless day corresponding to halfway between mid-veraison and harvest.

	1200 h		**1600 h**		***P*-value**					
Treatment	**East Facing side**	**West Facing side**	**East Facing side**	**West Facing side**	**Treatment (T)**	**Side of the row (S)**	**Time of measurement (TM)**	**T x S**	**T x TM**	**S x TM**
Control	20.17 ± 1.11	19.46 ± 0.94	25.86 ± 0.94	31.30 ± 3.13	0.431	<0.0001	<0.0001	0.034	0.176	<0.0001
Irrigation	21.05 ± 2.74	18.83 ± 0.71	24.75 ± 0.84	30.54 ± 4.08						

Conclusion

This study has enabled the characterization of the impact of on-vine grape drying with cutting of the fruit bearing cane (PES) on classical enological parameters and particularly on rotundone in wines for the two bunch populations (Spur and Cane) produced by the technique. Together with other findings previously reported on grape yield and fruit quality, our results show that the impact of the technique on rotundone accumulation was limited. In 2013, a slight reduction in this compound was observed for Cane. As PES induced an interruption of the sap flow, our results suggest that this key aroma compound in red wine is synthesized in the berries and is not translocated by the phloem from the leaves to the fruit. Our findings show that on-vine grape drying with cutting of the fruit bearing cane can be implemented without much affecting rotundone, on vineyard plots and/or during vintages with low water deficit in order to produce high quality (i.e. with higher phenolic concentrations) peppery wines. A viticultural system combining irrigation first and then PES investigated in 2014 with the aim of increasing rotundone while mitigating the negative effects of irrigation, allowed for the production of grapes with higher sugar concentration and red wines with higher rotundone, anthocyanins and TPI. Measurements of bunch surface temperature made on the system before the cutting of the canes indicate that the enhancement of rotundone production induced by irrigation is likely to be due to a direct rather than to an indirect effect due to an increase in leaf area leading to a cooler bunch microclimate. Our findings also suggest that bunch and acid rots don't induce the same natural response as powdery mildew, leading to the production of rotundone.

The results obtained may also be applicable to grapegrowers producing Shiraz, Gamay or other cultivars in which rotundone has been identified.

Acknowledgements

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Chapitre 4 :

Impact des techniques de vinification et des variables fermentaires sur la concentration des vins en rotundone

Compte tenu du caractère hydrophobe de la rotundone et de sa localisation pelliculaire (Caputi, et al. 2011), les **techniques de vinification** et les **variables fermentaires** favorisant la macération sont susceptibles d'influencer la concentration finale des vins en ce composé (Sacchi, et al. 2005).

Nos travaux ont permis de tester, en conditions de laboratoire, l'impact de la **durée** et de la **température de macération**, de l'**espèce de levures**, de l'utilisation d'**enzymes pectolytiques** et de techniques de vinification (**macération pré-fermentaire à froid**, **thermovinification**, **macération semi-carbonique**, **vinification en rosé**) sur la richesse en rotundone des vins (Article 8).

D'une manière générale, nous avons observé que la concentration en rotundone était faiblement corrélée à d'autres paramètres reflétant l'extraction des composés pelliculaires comme la teneur en **anthocyanes** (R2 = 0.48) ou l'**indice de polyphénols totaux (IPT)** (R2 = 0.28).

Si ces recherches nous ont permis d'identifier des leviers œnologiques faciles à mettre en œuvre pour réduire la concentration en rotundone dans les vins (e.g. utilisation de *Saccharomyces uvarum*, macération semi-carbonique), aucune des modalités testées, y compris l'augmentation de la température de macération et l'allongement de la durée de macération, ne se sont montrées efficaces pour maximiser son extraction à partir des pellicules.

Les vins **rosés** et **thermovinifiés** élaborés dans le cadre de cette étude présentaient des concentrations en rotundone particulièrement faibles, conséquence du retrait précoce des pellicules.

Article 8:

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Impact of winemaking techniques on classical enological parameters and rotundone in red wine at the laboratory scale

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Résumé : Ces travaux nous ont permis d'évaluer l'impact de plusieurs variables fermentaires et techniques de vinification sur la concentration en rotundone des vins de Duras N élaborés en conditions de microvinification (erlenmeyer d'1L). Les vins obtenus par thermovinification et vinification en rosé ont présenté les teneurs en rotundone les plus faibles. Aucune des modalités testées y compris l'utilisation d'enzyme pectolytique, l'allongement de la durée de macération à 14 jours ou l'augmentation de la température de macération à 30°C n'ont engendré de gain en rotundone par rapport à la modalité témoin vinifié à 25°C pendant 8 jours. Plusieurs pratiques faciles à mettre en œuvre comme la macération semi-carbonique ou l'utilisation de *Saccharomyces uvarum* ont été identifiées pour moduler la concentration en rotundone des vins à la baisse.

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Technical Brief

Impact of Winemaking Techniques on Classical Enological Parameters and Rotundone in Red Wine at the Laboratory Scale

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Abstract: The impact of fermentation variables (time and temperature of maceration, yeast species, addition of pectolytic enzymes) and winemaking techniques (cold soak, thermovinification, carbonic maceration and rosé vinification) on rotundone and classical enological parameters was investigated in triplicate in red wine made from Duras at the laboratory scale. Rotundone was badly correlated to anthocyanins and to Total Phenolic Index, some variables reflecting the extraction of skin compounds which suggests that the solubility of rotundone or its ability to bind to other materials may differ from anthocyanins and most of the grape proanthocyanidins. Compared to control wine, wines made from a rosé and a thermovinification treatment differed largely in their enological parameters. These two treatments involving a pre-ferment removal of skins resulted in the lowest wine rotundone concentrations, of ca. 20% and 13% respectively. The other treatments had a weak impact on the studied parameters and none of them, including the use of macerating enzymes and the increase in temperature or time of maceration, resulted in enhanced rotundone concentrations. Semi-carbonic maceration, fermentation with

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Saccharomyces uvarum or longer skin contact during extended fermentation resulted in wine with a significant decrease by ca. 20% in rotundone concentration, indicating practical opportunities for reducing pepper aroma in wine.

Key words: rotundone, red wine, winemaking techniques, fermentation variables

Introduction

Rotundone is an oxygenated sesquiterpene responsible for the peppery aroma in wines and was first identified in Shiraz grapes from Australia (Wood et al. 2008). Recent research into this aroma compound has been focusing on its biosynthesis (Drew et al. 2016, Takase et al. 2016), and also on the potential role of aerial oxidation of α-guaiene (Huang et al. 2014).

Viticultural and environmental factors driving the production of rotundone in grape berries have been widely investigated. Rotundone is most likely to accumulate in grapes in cooler vintages and in cooler vineyards (Caputi et al. 2011), and large spatial variations have been reported within a single vineyard (Geffroy et al. 2014, Scarlett et al. 2014, Zhang et al. 2015). Clonal differences in rotundone concentrations were identified among four certified clones of Duras; results from the same research suggest that rotundone might be involved in the vine's natural defence mechanisms in response to powdery mildew attacks (Geffroy et al. 2015a). Rotundone increases with the ripening of the fruit and is not impacted by grape thinning. Leaf removal has been shown to strongly lower rotundone, while irrigation enhanced its concentration (Geffroy et al. 2014).

So far within grape berries, rotundone has been only detected in the exocarp (Caputi et al. 2011, Siebert and Solomon 2011), yet the impact of maceration conditions and winemaking techniques on rotundone concentration in wine has been little studied to date. It appears most of the rotundone is extracted from the berries between days 2 and 5 of fermentation which corresponds to the period when ferments are very active and ethanol is increasing (Siebert and Solomon 2011). In another study, only 10% of rotundone present in grapes was extracted during fermentation, and only 6% was recovered in

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bottled wine. A significant amount of the compound can be lost during separation, filtration and fining processes, probably due to its hydrophobic nature (predicted Log K_{ow} = 4.98) and ability to bind to other materials (Caputi et al. 2011).

The effect of processing and fermentation parameters on the phenolic profile of red wine has been summarized in a review article (Sacchi et al. 2005). Fermentation temperature, maceration time, yeast selection, skin and juice mixing technique (punch-down or pump over) and their frequency, application of pectolytic enzymes together with winemaking techniques such as thermovinification, carbonic maceration and cold soak are known to impact the phenolic composition of red wines. Some of these compounds located in the berry skin or seeds such as high molecular weight proanthocyanidins are poorly water soluble (Cheynier et al. 2006). Therefore, winemaking techniques and fermentation variables are also likely to modulate rotundone extraction and the peppery character of red wine.

The purpose of the present work was to assess the impact of key fermentation variables (time and temperature of maceration, yeast species, addition of pectolytic enzymes) and winemaking techniques (cold soak, thermovinification, carbonic maceration) on rotundone concentration in red wine made from Duras at the laboratory scale. In order to get a good characterization of the experimental conditions, to identify possible side effects of the studied techniques and potential relationships between rotundone and other variables, classical enological parameters were determined in finished wines. A rosé vinification treatment was also included in the experimental design in order to study the extraction of rotundone associated with this process and to investigate whether this compound is likely to influence the aroma of rosé wines.

Material and Methods

Grapes and vineyard location. The experiment was carried out with grapes from *Vitis vinifera* L. cv. Duras collected in 2015 in the South West of France in the Gaillac Protected Designation of Origin (PDO) area. Grapes were sourced from an irrigated vineyard (lat. 43° 50′ 37‴ N; long. 01° 51′ 20‴ E)

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planted in 1999 with clone designated number 554 grafted on Gravesac rootstock. The vineyard with 2.20 m × 1 m vine spacing and moderate crop yields (6–8 t/ha) was trained with vertical shoot positioning on a single Guyot pruning system. Grapes were hand harvested on 16 September in 2 cases of 20 kg each. Then they were destemmed manually, mixed gently to constitute twenty-seven homogenous lots of 800 g.

Fermentation variables and winemaking techniques. Grapes were processed and fermented in the Institut Français de la Vigne et du Vin experimental cellar (Lisle Sur Tarn, France). Nine treatments were investigated in triplicate in 1 L Erlenmeyer: control vinification at 25°C for 8 days (Control), pectolytic enzyme addition (Enzyme), cold soak (Cold), thermovinification (Thermo), vinification at 30°C for 8 days (30°C), vinification at 25°C for 14 days (14 days), semi-carbonic maceration (Carbonic), rosé vinification (Rosé) and fermentation with *Saccharomyces uvarum* (Uvarum). For all the studied treatments with the exception of *Carbonic*, the 800 g berry samples were crushed and poured into a 1 L Erlenmeyer flask (with lid), and sulfur dioxide using a 10% bisulfite liquid solution was added (40 mg/L).

For *Control*, *Enzyme*, *30°C*, and *14 days* the musts were inoculated after 1 h with 200 mg/L rehydrated active dried *Saccharomyces cerevisiae* yeast (Lalvin Rhône 2056®, Danstar Ferment AG, Zug, Switzerland) and 300 mg/L of diammonium phosphate were added. Grapes collected on the experimental plot were usually poor in yeast assimilable nitrogen (typically less than 100 mg/L) and this addition rate of diammonium phosphate should allow the nitrogen to surpass 150 mg/L and to avoid stuck and sluggish fermentations. Just after the yeast addition, pectolytic enzymes (Lafase® HE Grand Cru, Laffort, Floirac, France) described by the provider to enhance the extraction of skin compounds were added for the *Enzyme* treatment at the rate of 40 mg/kg. While the *Control* and *Enzyme* treatments were fermented at 25°C for 8 days, post-fermentation maceration was extended by another 6 days to 14 days at the same temperature for the *14 days* treatment. The *30°C* vinification was carried out at 30°C for 8 days. For *Uvarum*, the vinification was conducted as in the control treatment with the exception of the yeast that was replaced by *Saccharomyces uvarum* (Velluto $_{\text{BMV58}}$™, Danstar Ferment AG, Zug, Switzerland). For

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Cold, grapes were cooled down at 4°C after crushing and maintained at this temperature for 72 h. At the end of that period, the flasks were warmed up to 15°C, inoculated with the control yeast, and then fermented like the control vinification for 8 days at 25°C. At the end of the maceration period, wines from these six treatments were pressed under controlled conditions (200 kPa for 2 min) using a Para Press laboratory press (Paul Arauner GmbH, Kitzingen, Germany). The amount of must and of skins was measured to determine skin-to-juice ratio at pressing (%).

For *Rosé*, maceration on skins for 6 hours at 18 °C was carried out after crushing. Grapes were then pressed and the amount of juice and of skins was determined. The musts were centrifuged (14 000 × *g* for 6 min), yeast and nutrient additions were performed with Lalvin Rhône 2056® at 200 mg/l and diammonium phosphate at 300 mg/L respectively. The musts were then fermented at 18°C for 12 days.

The *Thermo* treatment was performed using a water bath system. The 1L Erlenmeyer flasks containing the crushed and sulfited grapes were submerged into heated water for 2.5 h. The three flasks were closed by a lid during the heat treatment to avoid any water evaporation. The temperature was carefully monitored during this period and the grapes were mixed every 10 min to homogenize their temperature. The rise in temperature of the grapes from room temperature up to 70°C took exactly 30 min. Therefore, the effective heating time at 70°C was 2 h; a temperature/time ratio commonly used at wineries. Then, the grapes were pressed while at high temperature, the amount of wine and of skins was measured and the musts were centrifuged (14 000 × *g* for 6 min) before addition of yeast (200 mg/L) with Lalvin Rhône 2056®. Then, *Thermo* wines were fermented at 18°C for 12 days.

For *Carbonic*, the samples were heated to 30°C before crushing. 200 g of berries were crushed, placed in the bottom of the Erlenmeyer flasks and was inoculated with Lalvin Rhône 2056® yeast at 200 mg/L. This operation simulated the natural crushing that takes place under the weight of grapes in natural cellar conditions. During the filling of the flask with the rest of the whole berries, exogenous carbon dioxide was used and maintained for 36 hours. Closed flasks were kept at 30°C for 8 days. After pressing

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and determination of the amount of wine, 300 mg/L of diammonium phosphate were added and the wines were fermented at 18°C for 12 days without receiving any additional yeast addition.

Each fermentation flask was weighed daily to follow the fermentation kinetics and mixed to promote consistent extraction of the rotundone from the berry skins especially for the treatments fermented with the pomace. At the end of the fermentation/maceration period, wines were centrifuged (14 000 × *g* for 6 min) and received a sulfite addition of 80 mg/L before being stored at 4°C or below until analysis.

Nitrogen content, conventional enological parameters and rotundone analyses. For each replicate of the *Control* treatment and for each replicate of the *Thermo* treatment, 10 mL of grape musts were sampled just after crushing and just after pressing at hot temperature respectively. Musts were centrifuged (14 000 × *g* for 6 min) and enzymatic determinations of ammonium based on its reaction with α-ketoglutaric acid were conducted using a kit provided by Thermo Electron Corporation (Waltham, USA) and a Konelab Arena 20 sequential analyzer (Thermo Electron Corporation, Waltham, USA). The amino acid analysis was carried out using the method proposed by Dukes and Butzke (1998).

Conventional enological parameters were determined for the wines after one week. Alcohol content was measured using an Alcoquick L200 infralyser (Unisensor, Karlsruhe, Germany) and pH with a Titromatic pHmeter (Hachlange, Düsseldorf, Germany). Total acidity was measured according to the OIV method (2009). A Konelab Arena 20 sequential analyzer (Thermo Electron Corporation, Waltham, USA) using enzyme kits provided by several suppliers was used to determine volatile acidity, fructose and glucose (Megazyme, Wicklow, Ireland); and malic acid (Thermo Fisher Scientific, Waltham, USA). Potassium determination was done by flame photometry (Bio Arrow, France) according to the OIV method (2009) and tartaric acid determination by colorimetric titration (Hill and Caputi, 1970). Anthocyanins and Total Phenolic Index (TPI) were quantified according to the techniques described by Ribéreau-Gayon and Stonestreet (1965) and Ribéreau-Gayon (1970), respectively, using an Evolution 100

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spectrophotometer (Thermo Electron Corporation, Waltham, USA). All determinations were carried out in duplicate. Rotundone in wine was determined by solid phase microextraction-multidimensional gas chromatography-mass spectrometry (Geffroy et al. 2014).

Yeast identification. Four day after yeast inoculation, for each replicate of the *Control* and *Uvarum* treatments 20 mL of fermenting must were sampled using sterile equipment and stored overnight at 4°C. Then the supernatant was removed, 10 mL of a 0.9% sodium chloride solution and 5 mL of glycerol (purity 98%) were added and, the samples were stored at -20°C until analysis. Differentiation between yeast species was carried by PCR–RFLP analysis of the rDNA ITS region according to the protocol proposed by McCullough et al. (1998). As control, the *Saccharomyces cerevisiae* strain was identified by PCR-amplification of DNA delta sequences (Legras and Karst 2003).

Statistical analysis. Statistical analyses were conducted with Xlstat software (Addinsoft, Paris, France). All the analytical data and rotundone content of the wines were subjected to a one-way analysis of variance (ANOVA) treatment. Fisher's least significant difference (LSD) test was used as a post-hoc comparison of means at $P < 0.05$. A principal component analysis (PCA) was performed on classical enological and skin-to-juice ratio data using significant variables ($P < 0.05$) and rotundone concentration as an additional data. Regression analyses of the linear correlations between rotundone concentration and other variables were also carried out.

Results and Discussion

The sum of amino acids and ammonium concentrations in the *Control* and *Thermo* musts was 90 ± 4 mg/L and 168 ± 9 mg/L respectively which is consistent with previous results highlighting large gains in must nitrogen when grapes are pressed at hot temperature (Geffroy et al. 2015b). For *Control* and the other treatments with the exception of *Thermo*, this content was increased by 72 mg/L thanks to the addition of diammonium phosphate which contains 24% of nitrogen. Therefore, no bias due to variable

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content of nitrogen among the treatments was introduced in our experimental design. For all the experimental wines, the concentration in glucose and fructose at measurement did not exceed 0.5 g/L.

The PCA plot (Figure 1) shows that the *Rosé* and *Thermo* treatments had the largest impact on the enological parameters while the other studied treatments appeared to have a limited effect. In accordance with the PCA results, regression tests (n = 27) showed that rotundone was badly correlated to variables reflecting the extraction of skin compounds such as anthocyanins (r^2 = 0.48) and TPI (r^2 = 0.28). This suggests that the solubility of rotundone or its ability to bind to other materials may differ from anthocyanins and most of the grape proanthocyanidins

The detailed impact of the studied techniques on classical enological parameters, skin-to-juice ratio at pressing and rotundone are presented in Table 1 and Figure 2.

For *Enzyme*, the pH was slightly reduced in comparison with the control vinification which might be the consequence of a higher extraction of organic acids contained in the berry exocarp even if this could not be confirmed by determination of tartaric and malic acids. The pectin degradation induced by the macerating enzymes is known to maximize juice yield and as consequence a decrease in the skin-to-juice ratio was observed. Surprisingly, total phenols (TPI) and rotundone were not impacted by the enzyme addition. A significant gain in skin compounds (i.e. proanthocyanidins and rotundone) would have been expected as in the majority of studies, pectinases seem to increase extraction of hydrophobic molecules without enhancing the extraction of water soluble compounds such as anthocyanins (Sacchi et al. 2005).

Cold soak had no effect on anthocyanins and TPI which is consistent with most of the results reported on this technique (Sacchi et al. 2005). As cold soak is provoking a larger extraction of compounds soluble in aqueous phase, rotundone was not expected to be impacted by this technique due to the requirement for ethanol in its solubilisation. A gain in pH was noticed as a probable consequence of a

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modification in the acid-base balance induced by a larger aqueous extraction of potassium from the pericarp tissue, although the potassium concentration did not differ statistically from the control.

In accordance with research by Geffroy et al. (2015b), an increase in alcohol content, pH, tartaric acid, malic acid and volatile acidity was noticed for the *Thermo* treatment as a consequence of a larger extraction of amino and organic acids from the berry exocarp when pressing the grapes at hot temperature. The modification of the acid-base balance also provoked a clear decrease in total acidity. The ethanol concentration is particularly high for this treatment and cannot be explained by reduced evaporation of ethanol during the alcoholic fermentation carried out at 18°C rather than at 25°C. The gain in amino acids that can reach +200% for a 2-hour heat treatment at 70°C (Geffroy et al. 2015b) might also have contributed to higher ethanol yields (Albers et al. 1996). Results obtained for TPI and anthocyanins are decoupled, with higher TPI being observed for the *Thermo* vinification while for anthocyanins the highest concentrations were recorded in the control treatment. This suggests either a modification of anthocyanins structure or degradation under the pre-fermentation heat treatment. Among the studied treatments, *Thermo* wines had one of the lowest rotundone concentrations. A loss in rotundone through evaporation or thermal degradation is unlikely for this treatment as the compound is known to have a low volatility (Siebert et al. 2008) and to be stable (Herderich et al. 2013). Therefore, our results tend to demonstrate that rotundone is less heat-extractable than most of the wine proanthocyanidins in aqueous media.

The impact of maceration parameters (time and temperature of maceration) on TPI and especially on rotundone reflected in wine from the *30°C* and *14 days* treatments are unexpected. The literature usually supports the belief that the extension of the time and the increase in the temperature of maceration enhance extraction of the skins and seeds (Sacchi et al. 2005). While TPI was not significantly impacted by the two treatments, anthocyanins and rotundone concentrations were reduced for both treatments. As proposed by Gao et al. (1997), the reduction in anthocyanins observed at 30°C might be the consequence

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of an increase in polymeric pigments improving the overall color stability. In most of the previous studies, measurements were made in bottled wines corresponding to an unclear period after alcoholic fermentation (AF). In our experimental conditions, analyses were carried out one week after the end of AF. This decrease might not have been observed after a longer period, during which the loss in color due to instability of anthocyanins would have been more significant for the control treatment. For the *14 days* treatment, the loss in anthocyanins and rotundone might be due to a larger absorption by gross wine lees (tartrate and yeast biomass) that were settled when the pressing occurred. The two treatments also modified the acidity (titratable acidity and tartaric acid for *30°C* only) provoking a clear increase in pH which might be the combined effect of both larger extraction of tartaric acid and potassium and precipitation of potassium tartrate prior to analysis during the week of storage at 4°C.

Wine produced by the semi-carbonic maceration metabolism had significantly smaller concentration of alcohol, anthocyanins, titratable acidity and a higher pH in accordance with previous studies (Flanzy et al. 1987, Miller and Howell 1989). According to these authors, the decrease in ethanol production can be explained by the inversion of the Krebs cycle with some substrate of ethanol being converted to succinate and by the metabolization of a portion of the sugar to Krebs cycle intermediates. Under our vinification conditions (30°C/8 days) wines elaborated under carbonic maceration presented a similar TPI in comparison with the control. Most of the observations made on the technique (Sacchi et al. 2005) showed that the extractability of the phenolic compounds strongly depends on cultivars and conditions of implementation. In comparison with control, rotundone concentration was reduced by 23% for this treatment.

The *Rosé* treatment showed a higher concentration in tartaric acid and a lower pH which reflects a lesser extraction of potassium from the skin and consequently a reduced precipitation of potassium bitartrate. The smaller concentration of malic acid in wine for this treatment suggests a lesser extraction of malate from the pericarp. The difference in compartmentation between tartarte and malate at ripeness

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(Iland and Coombe 1988) with concentrations in malate being proportionally larger in the skin than in the flesh might explain the reasons why this decrease was not observed for tartrate. Considering our experimental conditions (levels of pH and sulfur dioxide addition), the lower concentration in malic acid for this treatment is unlikely to be due to a partial spontaneous malolactic fermentation. As expected, TPI, anthocyanins and rotundone were lowered for the *Rosé* treatment due to the early removal of grape skins prior to alcoholic fermentation. The rotundone in *Rosé* wines represented on average 13% of the concentration found in the *Control*. As the compound has an aroma detection threshold of 8 ng/L in water and 16 ng/L in red wine (Wood et al. 2008), this indicates than in most cases, rotundone is not likely to have a large sensory contribution to the aroma of rosé wines, unless it is present in unusually high concentrations in grapes sourced for a rosé wine.

PCR tests confirmed the fermentation by the Lalvin Rhône 2056® and *Saccharomyces uvarum* yeasts in the *Control* and *Uvarum* treatments respectively. Rotundone, anthocyanins and pH were significantly impacted by the *Uvarum* treatment relative to the control treatment. The decrease in anthocyanins and rotundone previously described by Tosi et al. (2009) for anthocyanins could be the consequence of a larger adsorption by the *Saccharomyces uvarum* yeast species. Despite similar concentrations in potassium, malic and tartaric acids, an increase in pH was noticed in wines fermented with *Saccharomyces uvarum* in comparison with the control treatment. This species is known to be cryotolerant, to produce high levels of glycerol, 2-phenylethanol and its acetate, and small amount of acetic acid (Masneuf-Pomarède et al. 2010). The differences in pH might be due to differences in metabolism of secondary products (including acids) between the two species. However, under our experimental conditions, volatile acidity was not impacted by the *Uvarum* treatment.

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Conclusion

This study has enabled to characterize the impact of winemaking techniques and fermentation variables on classical enological parameters and rotundone concentration in wine at a laboratory scale. Compared to control wine, the *Rosé* and *Thermo* treatments had the largest impact on enological parameters. These two treatments involving a pre-ferment removal of skins resulted in the lowest wine rotundone concentrations, of ca. 20% and 13% respectively. The other treatments had a weak impact on the studied parameters and none of them, including the use of macerating enzymes and the increase in temperature or time of maceration, resulted in enhanced rotundone concentrations in comparison with the control treatment. This means that efforts to maximize rotundone in wine have to be conducted in vineyards. Pre-fermentation heat treatment and semi-carbonic maceration were not favorable to enhancing rotundone concentrations in wine. This is an important observation as these techniques are often applied with Gamay, a cultivar where rotundone makes an important contribution to wine aroma (Geffroy et al. 2014). The rotundone in rosé wines represented only 13% of the concentration found in the control which indicates that in most case, rotundone is not likely to have a large sensory contribution to the aroma of rosé wines. *Saccharomyces uvarum* had a small, but significant impact on rotundone in wine and the use of this yeast species may be a practical approach for lowering rotundone in wine.

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Table 1 Impact of winemaking techniques on conventional enological parameters measured in wines and skin-to-juice ratio at pressing. Means followed by the same letter within a row are not significantly different at $p \leq 0.05$ by LSD test.

Parameter	Control	Enzyme	Cold	Thermo	30°C	14 days	Carbonic	Rosé	Uvarum
Alcohol content (% vol.)	12.7[a] bc	12.6 bc	12.4 cd	13.4 a	12.3 cd	12.8 b	12.1 d	12.9 b	12.5 bcd
Titratable acidity (g/L tartaric acid)	6.85 ab	7.02 a	6.90 ab	6.18 c	6.46 c	6.55 bc	6.39 c	6.55 b	7.06 a
pH	3.57 d	3.52 e	3.60 c	3.72 a	3.65 b	3.67 b	3.70 a	3.43 f	3.62 c
Tartaric acid (g/L)	1.76 de	1.71 de	1.92 bc	2.15 a	1.39 f	1.82 cd	1.95 b	2.10 a	1.69 e
Malic acid (g/L)	3.74 b	3.76 b	3.86 ab	3.91 a	3.71 b	3.77 b	3.73 b	3.34 c	3.75 b
Volatile acidity (g/L acetic acid)	0.33 b	0.13 b	0.30 b	0.56 a	0.47 b	0.23 d	0.30 b	0.35 b	0.30 b
Potassium (g/L)	1.43 a	1.44 a	1.56 a	1.45 a	1.46 a	1.52 a	1.57 a	1.12 b	1.56 a
Total Phenolics Index (TPI)	51.6 bc	54.9 ab	48.6 c	60.2 a	45.8 c	46.0 c	56.3 ab	11.1 d	46.3 c
Anthocyanins (mg/L)	722 a	722 a	718 a	666 b	618 c	600 c	600 c	79 d	626 c
Skin-to-juice ratio at pressing (%)	24.6 d	18.5 e	24.8 d	47 c	23.3 d	23.4 d	54.8 b	65.6 a	25.5 d

[a]Mean of three replicates.

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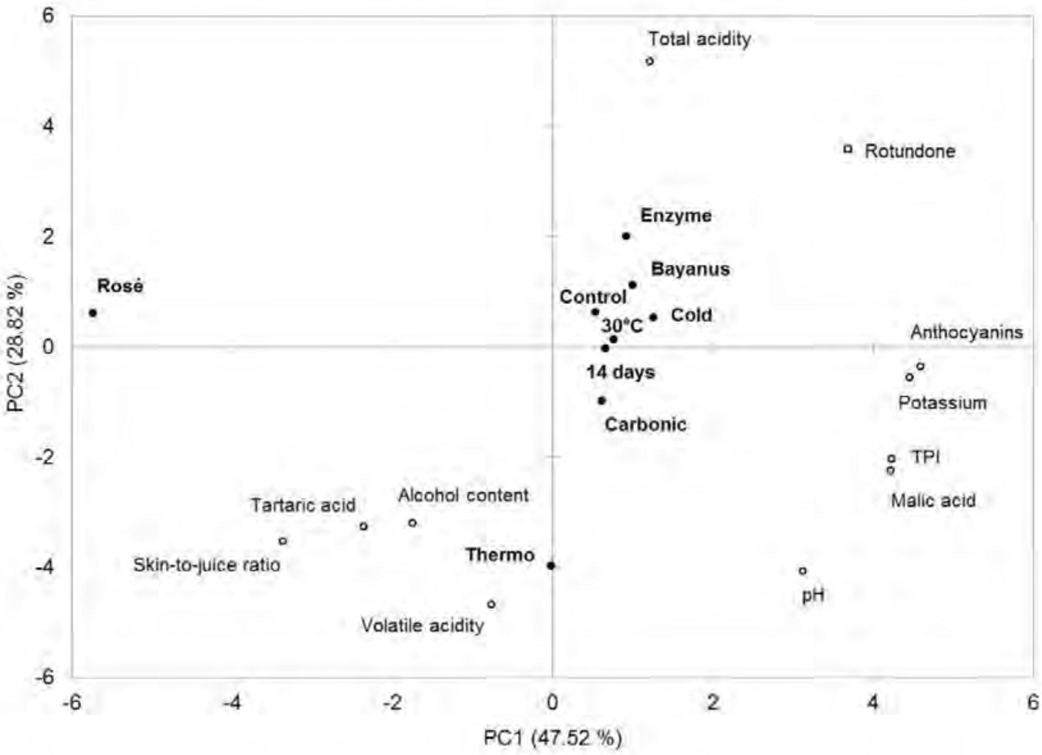

Figure 1 Factor loadings and scores for a principal component analysis (PCA) performed on the classical enological parameters and rotundone concentration as an additional data (square marker) for the 9 studied treatments.

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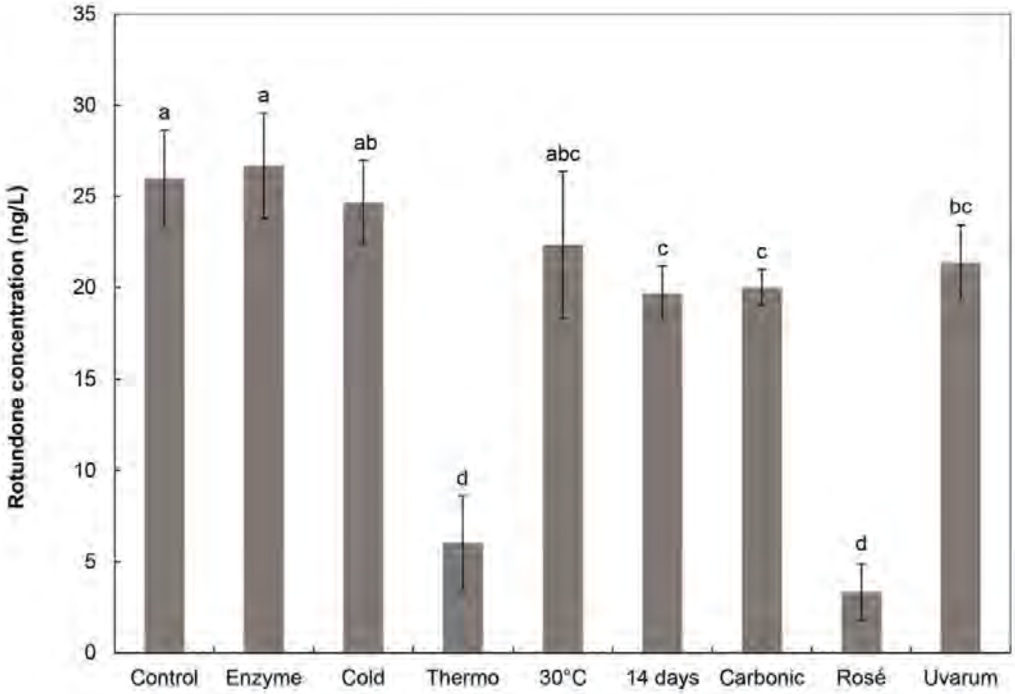

Figure 2 Impact of winemaking techniques on rotundone in wines. Different letters indicate means significantly different at $p < 0.05$ by LSD test.

Chapitre 5 :

Discussion générale et perspectives

1. Aspects sensoriels et appréciation par le consommateur

1.1 Relation entre concentration en rotundone et intensité des notes poivrées

Nos travaux ont permis d'établir une corrélation positive entre l'intensité poivrée à la dégustation et la concentration en rotundone de 21 vins de Gamay N (Article 1). Le coefficient de détermination obtenu au sein de notre échantillon (R2 = 0.66) est du même ordre de grandeur que ceux observés dans des études précédentes (0.50<R2<0.80) visant à mettre en relation concentrations en molécules aromatiques et données sensorielles (Lund, et al. 2009; Roujou de Boubée 2000; Vilanova, et al. 2010).

Cependant, la relation est moins forte que celle obtenue récemment par Homich, et al. (2017) sur Noiret N pour la rotundone (R2 = 0.79). Dans cette dernière étude, les vins ont tous été élaborés par vinification traditionnelle avec des températures de macération comprises entre 20°C et 25°C. Notre échantillon comprend 9 cuvées possédant une proportion de vins élaborés à l'aide de techniques de vinification alternatives (macération semi-carbonique ou thermovinification). Ces techniques sont connues pour favoriser l'expression fruitée des vins et l'obtention de teneurs particulièrement élevées en esters, acétates et acides gras (Flanzy, et al. 1987; Geffroy, et al. 2015c). Dans un mélange, il a été démontré que si certains composés pouvaient imposer leur odeur, d'autres en revanche étaient peu perçus (Godinot 1999). Ainsi, des interactions perceptives sont susceptibles d'avoir légèrement perturbé la perception olfactive des notes poivrées par notre jury. Ces effets doivent avoir été d'autant plus marqués que la rotundone possède une faible volatilité en comparaison avec les autres composés d'origine fermentaire (Siebert, et al. 2008).

Il est possible que ces **interactions perceptives** soient davantage prononcées sur les vins élaborés par macération carbonique et quasi négligeables sur ceux issus de thermovinification en lien avec les concentrations attendues en rotundone. En effet, nous avons montré que si la macération semi-carbonique était plutôt favorable à l'extraction de la rotundone, les niveaux enregistrés dans les vins thermovinifiés étaient insignifiants (Article 8). Cependant, l'élimination des données associées aux cuvées élaborées par ces techniques alternatives ne permet pas d'améliorer le coefficient de détermination de notre modèle.

1.2 Anosmie à la rotundone

Le pourcentage de sujets anosmiques à la rotundone au sein de notre population **(31%)** (Article 2) est supérieur à celui observé en Australie (20 à 25%) dans le cadre des premières études sensorielles (Wood, et al. 2008). L'anosmie spécifique, qui désigne la capacité réduite d'un sujet à percevoir une molécule aromatique donnée, a été largement documentée notamment pour l'androstérone, une hormone stéroïdienne à l'odeur musquée produite par les mammifères. Des différences de perception ont été décrites entre les sujets européens, asiatiques et australiens pour ce composé (Wysocki et Gilbert 1989), ce qui tendrait à corroborer l'hypothèse selon laquelle la proportion d'anosmie à la rotundone diffère entre les populations françaises et australiennes.

Nous avons également mis en évidence que l'âge avait un impact sur la capacité à percevoir la rotundone, avec une proportion supérieure d'anosmiques chez les **panélistes âgés de plus de 55 ans** (Article 2). Ces constations sont en accord avec d'autres travaux soulignant un déclin plus rapide, à partir de la cinquantième année, de la capacité à percevoir une molécule pour laquelle une anosmie spécifique a été reportée (Wysocki et Gilbert 1989). Dans le cadre d'un travail dirigé visant à illustrer les tests de discrimination, nous avons eu l'opportunité de tester, dans des conditions similaires à celles de l'étude précédente, l'anosmie à la rotundone de 200 étudiants âgés de 19 ans environ. Dans cette population très jeune, le pourcentage d'anosmiques n'a pas dépassé 10%. Compte tenu des faibles effectifs dans cette catégorie d'âge lors de l'étude visant à déterminer un CRT pour la rotundone (Article 2), cette tendance n'avait pas pu être dégagée. Si les anosmies spécifiques sont censées refléter des polymorphismes dans les gènes codant pour les récepteurs olfactifs, ces éléments laissent suggérer l'existence d'autres **mécanismes de régulation notamment épigénétiques**.

Chez les panélistes anosmiques, le fait que l'addition la plus faible (25 ng/L) ait provoqué un stimulus conduisant au rejet de l'échantillon complémenté mérite quelques éléments d'explication. Parmi les 15 panélistes anosmiques qui ont préféré le vin témoin pour cette série, 47% et 27% d'entre eux ont mentionné, dans l'espace réservé à des commentaires libres, qu'il trouvait le vin enrichi respectivement moins fruité et plus acide. Il est bien connu que la plupart des molécules aromatiques sont capables d'induire des **sensations trigéminales** détectables par les sujets anosmiques (Doty, et al. 1978). Même si nos observations suggèrent que la rotundone est susceptible d'induire ce type de stimulus, les raisons pour lesquelles ce phénomène n'a pas été observé aux concentrations supérieures restent inconnues.

Il a été mis en évidence que le 2,4,6-trichloroanisole (TCA), même à une très faible concentration, était capable d'atténuer la **transduction du signal olfactif** sans générer d'odeur (Takeuchi, et al. 2013). Un partitionnement de la molécule de TCA à l'intérieur de la bicouche lipidique, en lien avec son caractère lipophile, permettrait d'expliquer cette suppression. Etant donné que la rotundone est elle-même lipophile (Caputi, et al. 2011), un mécanisme similaire pourrait être impliqué. Ce phénomène étant dépendant de la concentration (Takeuchi, et al. 2013), on peut supposer que la concentration en rotundone dans les vins de la première série n'était pas suffisante pour inhiber le signal, alors que cette inhibition était effective dans les trois séries de vin plus riches en rotundone qui ont suivi.

1.3 Perspectives

Même si la contribution de la rotundone à l'arôme poivré des vins ne semble aujourd'hui faire aucun doute, il est légitime de se demander si ce composé en est l'**unique contributeur**. En effet, d'autres molécules pourraient également participer à cette perception. Avant la découverte de la rotundone en 2008 (Wood, et al. 2008), les notes aromatiques caractéristiques du poivre étaient attribuées à une cohorte de sesquiterpènes (Jagella et Grosch 1999). Récemment, Zhang, et al. (2016a) ont montré que tout comme l'α-guaiène et la rotundone, d'autres sesquiterpènes (e.g. clovène, calaménène, α-humulène, épi-zonarène et δ-cadinène) s'accumulaient au cours de la dernière phase de la maturation des raisins. Ces sesquiterpènes, dont les niveaux dans le raisin et le vin sont susceptibles d'être impactés par les mêmes facteurs environnementaux et d'être corrélés à la rotundone, pourraient agir de manière synergique.

Cependant, cette hypothèse reste peu probable. Dans le cadre de nos recherches visant à déterminer un seuil de rejet pour la rotundone, nous avons conçu un itinéraire cultural permettant de minimiser les teneurs en rotundone dans un vin rouge de Duras N (Article 2). La concentration en rotundone de ce vin de base ne dépassait pas 10 ng/L. Lors d'une dégustation informelle réalisée auprès d'un panel composé de quelques dégustateurs, son expression particulièrement fruitée a été jugée comme atypique de la variété. Ce même vin possédait, selon ce même panel et après ajout de 100 ng/L de rotundone, une typicité extrêmement proche de celle des vins de Duras N.

Une étude plus formelle mériterait d'être conduite pour vérifier ces allégations. Cette dernière pourrait prendre la forme d'une **reconstitution aromatique**. Cette approche popularisée par Ferreira, et al. (2002) est inspirée des travaux de Grosch (2001). La démarche part du constat que l'impact d'une molécule reste difficilement prévisible par les techniques classiques dans un mélange complexe, du fait d'interactions physico-chimiques. Elle repose sur la préparation de modèles d'arômes obtenus en mélangeant les composés aromatiques purs, dans les proportions retrouvées dans les aliments. En étudiant l'impact de l'élimination d'un composé sur les caractéristiques sensorielles du modèle (souvent dénommé **test d'omission**), il est possible d'obtenir une preuve irréfutable de la contribution sensorielle de ce composé.

La réalisation d'un test d'omission pour la rotundone, associé à un traitement séparé des données en fonction de la capacité des panélistes à percevoir la molécule, pourrait permettre d'évaluer son impact sensoriel chez les sujets anosmiques. Ces questions font actuellement l'objet d'une étude menée par l'INP-PURPAN en collaboration avec le Laboratoire de Génie Chimique de Toulouse. Le projet comprend 3 phases : 1) identification de la meilleure matrice (vins synthétiques, vins désaromatisés par évaporation sous vide puis passage sur résine, ou par traitement au CO_2 supercritique), 2) optimisation du nombre de molécules dans le modèle sur la base de leur Odour Activity Value (OAV) défini par la ratio entre concentration et seuil de perception (OAV>0.5, OAV>1, OAV>2, OAV>5 et OAV>10), et 3) mise en œuvre des tests d'omission sur le meilleur modèle, en utilisant la méthode du Profil Pivot© (Thuillier, et al. 2015).

2. Facteurs viticoles et environnementaux

2.1 Clones et oïdium

Les concentrations en rotundone anormalement élevées observées en 2014, un millésime marqué par de forts dégâts d'oïdium (*Erysiphe necator*), et l'existence d'une corrélation entre l'intensité de ces dégâts et les niveaux de rotundone (Article 3), suggère que la molécule pourrait être impliquée dans les **mécanismes de défense** vis-à-vis de ce champignon notamment via la voie du mévalonate. En effet, les interactions hôtes/pathogènes ont été caractérisées pour l'oïdium chez *Vitis pseudoreticulata*, une espèce de vigne asiatique résistante (Weng, et al. 2014). Il a été démontré que l'infection des feuilles par ce champignon induisait l'expression différentielle de 6541 gènes dont un grand nombre associé à la voie du mévalonate, impliquant l'acide salicylique et l'acide jasmonique. L'une des seules études réalisées sur *Vitis vinifera* a mis en évidence que les concentrations en acide salicylique augmentaient lors d'une infection par ce pathogène. Cependant, la voie du MVA n'est certainement pas la seule impliquée puisqu'il a été mis en évidence que la pulvérisation exogène d'acide jasmonique sur grappes (Article 5) et d'acide jasmonique ou d'acide salicylique sur feuilles (Zhang, et al. 2016b), n'avait aucun impact ou un impact très limité sur la production de rotundone. La synthèse des sesquiterpènes mobilise également la voie du MEP avec des exports possibles entre chloroplaste et cytoplasme. Des travaux complémentaires visant à étudier l'impact d'une infection d'oïdium sur l'expression des gènes impliqués dans les voies du MVA et du MEP permettrait de mieux appréhender les mécanismes conduisant à la production de rotundone.

Il est important de souligner que les mécanismes en jeu semblent être **systémiques** puisque les vins expérimentaux ont été élaborés à partir de baies saines. Même si aucune infection par l'oïdium n'a pu être observée au cours de l'autre année de suivi du dispositif, le fait qu'aucune interaction *millésime x clone* ne soit observée, laisse supposer que les différences clonales de sensibilité à l'oïdium pourraient permettre d'expliquer les différences de concentrations en rotundone observées entre les clones.

Si des différences de sensibilité clonale ont été précédemment décrites pour le mildiou de la vigne (*Plasmopara viticola)* (Boso, et al. 2004), il s'agit, à notre connaissance, de la première fois que de telles différences sont reportées pour l'oïdium. Compte tenu de la structure de notre dispositif expérimental composé de 4 rangs adjacents, il n'est pas à exclure que les écarts observés reflètent une hétérogénéité de vigueur entre les rangs ou des variations dans la qualité de la pulvérisation. Cependant, les mesures d'indice de végétation par différence normalisé (NDVI) réalisés au sein du dispositif n'ont pas permis de discriminer les clones et aucun lien n'a pu être établi entre la séquence de plantation des 4 clones et l'intensité des dégâts d'oïdium.

Malgré les différences de richesse en rotundone observées entre les clones, cette variable qualitative n'a pas été intégrée dans les meilleurs modèles construits pour prédire la concentration en rotundone des vins de Duras N du Gaillacois (Article 4). Ceci peut s'expliquer par le fait que certains clones (654 et 627) étaient sous-représentés au sein de l'échantillon composé uniquement de 10 parcelles.

2.2 Impact de l'effeuillage

L'incidence de l'effeuillage apparaît complexe puisque cette pratique peut avoir un effet dépréciatif sur la concentration des vins en rotundone (Article 5), aucun impact significatif (Article 6) voire même dans certaines situations favoriser son accumulation (Homich, et al. 2017). Il est difficile de dissocier les effets induits par cette pratique sur la **quantité de lumière** pénétrant dans la zone des grappes et la **température de surface** des raisins. L'impact le plus dommageable a été observé lorsque la technique a été mise en œuvre tardivement, à partir de la mi-véraison, sur les deux faces du rang (Article 5). Les conditions climatiques lors de cette étude étaient plutôt chaudes, avec des températures moyennes supérieures à 20.5°C au cours de la maturation (Article 5). Il est connu que l'exposition des grappes au rayonnement solaire peut augmenter la température de surface des baies de plus de 10°C par rapport à l'air ambient (Spayd, et al. 2002). On peut supposer que dans cette situation, l'effeuillage a contribué à bloquer dans les baies exposées la production de rotundone, dont la biosynthèse est inhibée à partir de 25°C (Zhang, et al. 2015a; Zhang, et al. 2015b). Les deux autres études sur l'effeuillage ont été réalisées dans des conditions beaucoup plus fraiches, avec des températures moyennes sur la période véraison-récolte oscillant entre 14.0°C et 15.6°C sur Noiret N (Homich, et al. 2017), et entre 18.1°C et 18.4°C sur Fer N (Article 6). Par ailleurs, l'effeuillage ne concernait dans ces études qu'une seule face du rang. Dans ces conditions, on peut émettre l'hypothèse que l'élévation de température de surface des raisins n'a pas été suffisante, notamment sur Noiret N, pour compromettre la biosynthèse de la rotundone. Par ailleurs, le fait qu'un gain significatif en rotundone ait été observé, lorsque les feuilles néoformés sont éliminées jusqu'à la récolte pour maintenir une bonne exposition des grappes au soleil (Homich, et al. 2017), suggère que la lumière est capable de stimuler la production de la rotundone. Cette hypothèse est cohérente avec nos conclusions mettant en évidence une contribution positive, du nombre d'heures d'ensoleillement et de l'irradiation moyenne au cours de la maturation, à un modèle de prédiction de la rotundone (Article 4).

Il a été proposé que les **blessures physiques** liées à l'effeuillage pourrait stimuler la production de rotundone (Homich, et al. 2017). Cette hypothèse semble peu probable car les blessures liées à l'**échardage** sont sans effet sur la production de rotundone (Article 6).

2.3 Irrigation, cumul de précipitations et statut hydrique

Nos travaux ont permis de mettre en évidence un effet positif de l'**irrigation** (Article 5, Article 7), du **cumul de précipitations** (Article 4) et du **statut hydrique** au niveau intra-parcellaire (Article 5) sur la concentration en rotundone des vins. Par ailleurs, l'importance du **bilan hydrique** a également été souligné par Zhang, et al. (2015b) pour expliquer les différences de concentration en rotundone entre 15 millésimes sur un vin de Syrah N australien. Cependant selon ces auteurs, le pourcentage de degré heures au-dessus de 25°C (DH25) est le principal déterminant de la rotundone dans les vins. Le fait que les variables hydriques n'aient pas une contribution plus importante dans la modélisation de la rotundone pour les vignobles australiens pourrait s'expliquer par un coefficient de variation interannuel plus faible pour ces paramètres, les parcelles d'étude étant la plupart du temps irriguées. Dans nos conditions expérimentales l'irrigation n'étant pas pratiquée, ces variables pourraient s'avérer bien plus discriminantes. En effet dans le cadre de nos recherches, la contribution des variables hydriques apparait supérieure à celle des variables thermiques puisque les millésimes les plus chauds et les plus arrosés au cours de la maturation présentent toujours des teneurs supérieures en rotundone (Article 5, Article 6). L'état hydrique de la plante n'a jamais fait l'objet de suivi dans le cadre des recherches conduites en Australie. Or, les différences de contrainte hydrique pourraient être la manifestation de différences topographique et de résistivité des sols au sein d'une même parcelle, des variables permettant d'expliquer les variations de concentration en rotundone au sein des vignobles australiens (Scarlett, et al. 2014). Il en de même pour l'hypothèse formulée par Vadakattu, et al. (2019), selon laquelle la variation de microbiome du sol, à travers la répartition et la diversité des populations bactériennes et fongiques, pourrait avoir un effet sur la concentration en rotundone des fruits. Cette hypothèse est quelque peu étonnante et il est plus vraisemblable que les variations de sol soient responsables de différences de microbiome et de statut hydrique, et que ce soit cette dernière composante qui ait un impact sur la production de rotundone. Il est intéressant de mentionner que Geffroy, et al. (2015b) ont montré que la rotundone était corrélée à la circonférence des troncs, un indicateur du niveau de contrainte hydro-azotée subi par la vigne depuis sa plantation (Tisseyre, et al. 2005) Ce paramètre étant *a priori* stable dans le temps, il est peu surprenant que les motifs de distribution spatiale de la rotundone présentent également une certaine stabilité temporelle en Australie (Bramley, et al. 2017)

Zhang, et al. (2015b) ont émis l'hypothèse que l'irrigation possède un **impact indirect** lié à un microclimat des grappes plus ombragé et plus frais, conséquence d'une vigueur supérieure de la plante. Cette hypothèse est peu envisageable dans nos conditions pédoclimatiques du Sud-Ouest de la France puisque la contrainte hydrique est en général peu limitante lors du cycle végétatif et du développement des rameaux. Ainsi, la vigueur est souvent déconnectée du statut hydrique (Article 4) et davantage dépendant de la nutrition azotée du végétal. Des mesures de température réalisées avec un thermomètre infra-rouge vont également dans ce sens, puisque l'irrigation ne semble pas avoir d'impact sur la température de surface des raisins (Article 7).

Cet **effet direct** de l'irrigation est d'autant plus révélateur que la rotundone a été quantifiée dans les vins, et non dans les raisins. En effet, l'irrigation avant véraison est susceptible d'induire une augmentation du volume de la baie et une dilution des composés pelliculaires. En modifiant l'état hormonal de la baie, notamment les teneurs en acide indole 3-acétique, en acide jasmonique, en acide salicylique et en acide abscissique (Niculcea, et al. 2014), l'irrigation pourrait favoriser la production de rotundone dans la baie pour les raisons discutées précédemment (cf 2.1).

2.4 Perspectives

2.4.1 Validation de nos hypothèses sur plante modèle et en conditions contrôlées

Nos travaux menés à l'aide d'une approche écophysiologique, ont permis d'émettre un certain nombre d'hypothèses concernant les facteurs environnementaux, biotiques ou abiotiques, impactant les niveaux de rotundone dans les vins. Afin de valider ces hypothèses, notamment celles concernant l'effet stimulant d'*Erysiphe necator* (Article 3), de l'irrigation (Article 5, Article 8) et de l'éclairement (Article 4), des études complémentaires **sur plante modèle et en conditions contrôlées,** mériteraient d'être conduites. Le modèle privilégié pourrait être une microvigne (Torregrosa, et al. 2019), un mutant naturel permettant d'avoir simultanément sur le même pied tous les stades phénologiques de développement de la partie fructifère ou une bouture fructifère (Ollat, et al. 1998).

Par ailleurs, les travaux menés aujourd'hui se sont tous intéressés à la rotundone stockée dans la plante ou à sa synthèse mais jamais à la fraction émise. Les sesquiterpènes, dont la fonction biologique est liée à leur **émission**, sont conservés dans des organes de stockage. Le suivi simultané de la synthèse de la rotundone, des fractions stockée et émise permettrait de mieux comprendre les mécanismes impliqués au niveau de la plante et d'appréhender le rôle de la molécule chez la vigne. Ces aspects font l'objet, depuis janvier 2020, d'une thèse de doctorat au sein de l'unité Physiopathologie, Physiologie et Génétique Végétale (PPGV) de l'INP-PURPAN et du Laboratoire de Chimie Agro-Industrielle. Les fractions émises seront quantifiées à l'aide d'un *selected ion flow tube – mass spectrometry* (SIFT-MS), une technique analytique récente permettant la quantification de composés présents dans un gaz à l'état de traces, de l'ordre du ppt (Smith et Španěl 2005).

2.4.2 Récolte sélective

Nos travaux (Article 5), tout comme ceux menés en Australie (Bramley, et al. 2017; Scarlett, et al. 2014; Zhang, et al. 2015a), ont mis en évidence une importante variabilité de concentration en rotundone au sein d'une même parcelle. Ces constations rendent envisageables, grâce à la mise en œuvre d'une **récolte sélective ou différentielle,** l'élaboration de vins au profil sensoriel distinct à partir de raisins récoltés au sein d'une parcelle.

Dans nos conditions culturales et pédoclimatiques, ces variations semblent être liées au niveau de contrainte hydrique. Pour accéder de manière indirecte à la concentration en rotundone via la spatialisation de la contrainte hydrique, des techniques d'**échantillonnage stratifié** ont été mises en œuvre (Geffroy, et al. 2015b). La variabilité d'architecture de la vigne au niveau intra-parcellaire a été évaluée grâce à des mesures de circonférence moyenne de tronc. Sur la base des trois classes de circonférence de tronc obtenues, six zones de suivi de 50 pieds dénommées « smart points » ont été définis et ont fait l'objet d'un suivi agronomique et œnologique. Les différences observées entre ces smart points sont très faibles d'un point de vue agronomique et peu d'indicateurs de la physiologie de la plante ont permis de discriminer le comportement de la vigne. Malgré cette faible variabilité pour la plupart des paramètres mesurés sur la vigne et les raisins, la concentration en rotundone est en moyenne 50% plus élevée dans les vins issus de la zone à forte circonférence de tronc. A la dégustation, les vins élaborés à partir de raisins récoltés sur les zones aux troncs plus développés présentent des notes poivrées plus prononcées. Ces constatations suggèrent que cet indicateur peut être utilisé afin d'approcher la distribution spatiale de la rotundone.

2.4.3 Stratégies d'adaptation au changement climatique

Les millésimes chauds et humides étant particulièrement défavorables à l'accumulation de la rotundone, l'**évolution climatique** par la diminution du régime des précipitations et l'élévation des températures attendus risque très fortement d'altérer ce potentiel aromatique. Cet effet risque d'être amplifié par l'avancement de la phénologie avec des maturations se déroulant dans des conditions plus chaudes. Si l'irrigation a démontré son efficacité pour favoriser l'obtention de vins pourvus rotundone, cette stratégie n'est pas durable sur le long terme. L'adaptation au changement climatique fait l'objet de nombreuses recherches, et certaines stratégies mériteraient d'être évaluées pour favoriser l'accumulation de la rotundone ou limiter sa dégradation.

Ainsi, des **pulvérisations d'un film blanc protecteur** à base de **kaolin** pourrait permettre de réduire la température de surface des raisins et de limiter le stress thermique (Shellie et King 2013). La **taille tardive** après débourrement, une pratique déjà expérimentée il y a plus de 50 ans (Antcliff, et al. 1957), refait parler d'elle aujourd'hui pour décaler la maturation des raisins vers des conditions plus fraiches (Friend et Trought 2007; Gatti, et al. 2016). Cependant, la phénologie n'étant retardée que de quelques jours, l'impact attendu de cette pratique sur la température moyenne au cours de la maturation devrait rester limité.

La piste du matériel végétal apparaît plus prometteuse. Il a été montré que la transpiration du greffon était régulée génétiquement par le **porte-greffe** (Marguerit, et al. 2012). Ces travaux ont également permis l'identification de régions chromosomiques (*quantitative trait loci* ou QTL) associés à ces traits, ouvrant la voie à de nouveaux programmes de sélection de porte-greffes adaptés à la sécheresse. En conférant une plus forte vigueur au greffon et en limitant le niveau de contrainte hydrique, ces porte-greffes du futur devraient être favorables à l'accumulation de la rotundone dans les raisins. Le **cépage** constitue un autre levier d'adaptation. La quasi-totalité des travaux menés aujourd'hui à l'échelle mondiale sur la rotundone concernent des variétés précoces (Syrah N, Duras N et Gamay N). La maturation de ces variétés intervenant dans des conditions généralement chaudes (de début août à mi-septembre dans l'hémisphère nord) et par conséquent défavorables à la production de la rotundone. L'identification de génotypes mûrissant tardivement (e.g. début octobre) et aptes à produire de la rotundone, devrait permettre de maîtriser le potentiel poivré.

3. Techniques de vinification et variables fermentaires

De manière plutôt inattendue, nos travaux ont montré que les vins macérés pendant 14 jours, possédaient une concentration en rotundone inférieure à ceux macérés pendant 8 jours (Article 8). Entre le 8ème et le 14ème jour de macération, le dégagement de CO_2 étant nul, les levures mortes sont susceptibles de sédimenter. Ces constatations suggèrent que la rotundone possède une **forte affinité avec les lies**. Cette hypothèse est renforcée par le fait que les vins fermentés avec *Saccharomyces uvarum* sont moins concentrés en rotundone (Article 8). Une capacité d'adsorption supérieure, notamment vis-à-vis des anthocyanes, a préalablement été décrite pour cette espèce de levure, (Tosi, et al. 2009). La validation de ces hypothèses pourrait permettre de valoriser la rotundone contenue dans les lies à l'aide de filtre rotatif sous vide ou de filtre presse. Des différences de capacité d'adsorption des anthocyanes ayant également été reportées chez *Saccharomyces cerevisiae* (Morata, et al. 2003), l'impact de plusieurs souches de cette espèce sur la concentration en rotundone des vins mériterait également d'être évalué.

Conclusion générale

Les travaux entrepris dans le cadre de cette thèse ont permis de nombreuses avancées sur la rotundone, le principal contributeur aux notes poivrées des vins rouges.

D'un point de vue sensoriel, nous avons mis en évidence l'existence d'une **corrélation entre la concentration en rotundone et l'intensité des notes poivrées à la dégustation**. Les études consommateurs ont permis d'identifier le profil de consommation des vins riches en rotundone. Nous avons également démontré, dans la plupart des cas, le **caractère positif** de la molécule en utilisant la méthodologie du seuil de rejet par le consommateur. Le fait que l'ajout de faibles quantités de rotundone provoque chez les anosmiques un stimulus conduisant au rejet de l'échantillon complémenté suggère que la molécule peut induire une sensation trigéminale et/ou être impliquée dans des mécanismes de suppression aromatique. A ce titre, ces résultats ouvrent la voie à de nouvelles recherches pour mieux appréhender les **mécanismes associés à l'anosmie à la rotundone**.

Nos recherches sur l'écophysiologie de la rotundone suggèrent que **la production de la molécule a lieu *in situ*** puisque la modification des relations de type source-puit et l'interruption du flux de sève sont sans effet sur son accumulation dans les vins. Ces travaux ont également montré que la production de la rotundone pouvait être stimulée par des **facteurs abiotiques** (quantité d'eau, niveau d'éclairement) et **biotiques** (infection par *Erysiphe necator*). *A contrario*, la pourriture grise (*Botrytis cinerea*) possède un effet préjudiciable sur la concentration des vins en rotundone. L'effet généré par les **apports d'eau** semble être davantage **un effet direct**, plutôt qu'un effet indirect induit par une modification du microclimat des baies puisque ces apports ne modifient pas la température de surface des raisins. Ces hypothèses mériteraient d'être validées en conditions contrôlées sur plante modèle.

Nos recherches ont permis d'identifier des **leviers viticoles** permettant de moduler la concentration en rotundone des vins. Ainsi la **date de récolte, le clone, l'effeuillage, l'irrigation ou la mise en œuvre de récolte sélective** sont autant de pratiques utilisables par les vignerons pour favoriser l'expression poivrée de leurs cuvées. Il est utile de mentionner que ces pratiques ont parfois induit une modification modérée de la concentration des vins en rotundone, de l'ordre de quelques dizaines de pourcents par rapport à la pratique témoin. Si ces différences ont pu être mises en évidence à l'aide des méthodes analytiques utilisées, elles n'auraient pas forcément été détectables sensoriellement par un jury entraîné. L'apport de la chromatographie en phase gazeuse couplée à la spectrométrie de masse a été particulièrement précieux dans le cadre de nos travaux. Cela signifie également qu'il peut s'avérer nécessaire, pour renforcer la typicité poivrée d'un vin, de combiner au sein d'un **itinéraire cultural optimisé** plusieurs pratiques à effet bénéfique que nous avons pu identifier.

Ces avancées sont d'autant plus déterminantes qu'aucune des **techniques de vinification** et des **variables fermentaires** testées, y compris l'utilisation d'enzyme pectolytique, l'allongement ou l'augmentation de la température de la macération, n'a permis de maximiser la rotundone dans les vins. Ces constatations suggèrent que la rotundone possède une **forte affinité** avec les lies et que la souche de levure, au sein de l'espèce *Saccharomyces cerevisiae*, est susceptible d'impacter la concentration en rotundone des vins.

Par la diminution du régime des précipitations et l'élévation des températures attendus, le **changement climatique** risque fortement d'altérer la typicité poivrée des vins, les millésimes chauds et humides étant particulièrement défavorables à l'obtention de vins riches en rotundone. L'irrigation a démontré son efficacité pour favoriser l'accumulation de la rotundone dans les vins. Cependant, cette pratique n'est pas envisageable sur le long terme compte tenu de la raréfaction de la ressource en eau. A ce titre, le **matériel végétal** et notamment le **cépage**, constitue l'une des pistes d'adaptation les plus prometteuses. La quasi-totalité des travaux menés aujourd'hui sur la rotundone concernent des variétés précoces qui mûrissent sous des conditions chaudes, et sont récoltées autour de la mi-septembre dans le Sud-Ouest de la France. L'utilisation de génotypes aptes à produire de la rotundone et mûrissant plus tardivement, lorsque les températures commencent à devenir plus fraiches, pourrait permettre de maîtriser ce potentiel poivré. L'exemple du Tardif N, une variété quasi-disparue, inscrite au Catalogue Officiel en 2017 et en cours de redéploiement dans le vignoble de Saint-Mont, tend à conforter cette hypothèse. Les vins de Tardif N expriment des notes poivrées particulièrement prononcées, même lors de millésimes chauds et secs comme 2017 ou 2018. En effet comme le laisse supposer son nom, la maturité de cette variété intervient à la mi-octobre, un mois après les autres variétés poivrées présentes sur le bassin Sud-Ouest.

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