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Evolution of phytosterols in Chardonnay grape berry skins during last stages of ripening

by

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S u m m a r y : This study presents the results of rapid phytosterols analysis in grape skins during last stages of ripening. The analysis is related to the evolution of sterol content by comparison with ripening degree on two vineyards of Chardonnay grape variety in Burgundy : Meursault 1er Cru and Hautes Côtes de Beaune. The characterization of sterols is realized by using combined gas chromatography-mass spectrometry from trimethylsilyl ethers of sterols. After optimization of extraction by azeotropic mixture (chloroform/methanol 2:1 v/v), the analysis allows to identify four sterols in grape skins : ß-sitosterol, campesterol, stigmasterol and lanosterol. In all the samples, ß-sitosterol is the major phytosterol (86 to 89 % of the total detected phytosterols). The evolution of phytosterols content during last stages of ripening shows a similar comportment of ß-sitosterol, campesterol and stigmasterol in grape skins : the maturation induces a loss of phytosterols in grape skins. An increase of phytosterol contents occurs at peak maturity and can be related with over-maturation phenomenon. The relationship between phytosterol content in grape skins and S/A ratio indicates a markedly negative correlation.

K e y w o r d s : sterols, ripening, grape skin, gas chromatography, mass spectrometry.

Evolution de la teneur en phytostérols dans les pellicules de Chardonnay au cours des derniers stades de la maturation

R é s u m é : Cette étude porte sur l'application d'une méthode rapide d'analyse des phytostérols dans les pellicules de raisins. Les stérols sont analysés sous forme d'ethers de triméthylsilyle par couplage CPG/spectrométrie de masse. Trois phytostérols sont ainsi identifiés et dosés : le campestérol, le stigmastérol et le ß-sitostérol. L'analyse des extraits lipidiques a permis d'identifier également le lanostérol. L'étude des phytostérols contenus dans les pellicules de raisins met en évidence une corrélation négative entre l'indice de maturité et la teneur en stérols, et cela dans les quatre à cinq dernières semaines qui précèdent les vendanges. Ce phénomène a été mis en évidence sur deux parcelles de Chardonnay en Bourgogne dans des conditions de maturation différentes.

Introduction

Qualitative and quantitative analysis of lipid components in grape varieties have been the object of extensive research, especially in leaves (BENTCHIKOU *et al.* 1993) and grape seeds (LAVAUD and CHERRAD 1980; CHERRAD and LAVAUD 1993). In addition to grape seeds, the cuticular wax of grape has been studied and the results have shown that the lipid fraction of grape skin was 0.32 %, expressed in fresh weight, whereas the pulp contained only 0.10 % (HIGGINS and PENG 1976). Analogous studies on grapes deal exclusively with their fatty acids composition divided in the groups of neutral lipids, phospholipids and glycolipids (BAUMAN *et al.* 1977; GALLANDER and PENG 1980). More recently, the distribution of fatty acids in several anatomical parts of the berries have been studied in Cabernet Sauvignon grape variety (MIELE *et al.* 1993). The main lipid fraction evidenced in grape skins was phospholipids. The neutral lipids fraction represented around 40 % of lipid content in grape skins. The major lipid components were polyunsaturated fatty acids, especially oleic, linoleic and linolenic acids.

Few of the many investigations of the evolution of grape constituents during ripening have related to lipids. BAUMAN *et al.* (1977) in American grape variety (Concord) then ROUFET *et al.* (1987) in Vitis vinifera have previously described the effects of maturation on the fatty acids content in grapes. During grape maturation, change in fatty acids level were low, except for linolenic acid, which decreased consistently. This loss was concerned with neutral and glycolipid fractions. If fatty acids composition during grape ripening has been studied , the evolution of phytosterols study has not been performed. In contrary, the study of sterols in different tissues of grapes has been already carried out (DAGNA *et al.* 1982; FREGA *et al.* 1982). The main phytosterol identified in grape skins was ß- sitosterol.

In the present study, unesterified and esterified phytosterols from grape skins were separated and identified by coupled GC-MS. A rapid method of sterol extraction from vegetal material was described. Sterols were analyzed during the last stages of ripening, five weeks before harvest. The relationship between phytosterol content in skins and ripening degree was established.

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Materials and methods

P l a n t m a t e r i a l : Chardonnay grape samples were taken weekly during the last period of ripening in 1991 in Burgundy vineyards at 2 plots: (1) Meursault Bouches Chères (Mersault 1er Cru) from September 3 to 24 (harvest); (2) La Grande Châtelaine (Hautes Côtes de Beaune) from September 3 to October 10 (harvest). Bunches were harvested at random in 5 different areas of the 2 plots; berries were separated and skins were taken, dried and kept at -18°C.

After pressing and centrifugation (4000 r/min for 5 min), sugar content (g/l) and total titratable acidity (expressed in g/l H_2SO_4) of pulp were determined by the methods recommended by O.I.V. The sugar/total titratable acidity ratio was calculated and has indicated the ripening degree.

From each sample, freezed grape skins were lyophilised (Leybold-Heraeus GT2) for 16 h. After lyophilisation, the homogeneous samples of dried skins were pulverized by successive fractions for 30 s. The powders of skins were kept at low temperature in vacuum-dessicator and under shelter from the light.

S t e r o l e x t r a c t i o n : The extraction of lipid compounds, especially unsaponifiable fraction, was performed as follows : 5 g of powder were suspended in 150 ml of redistilled Folch mixture (chloroforme: methanol, 2:1 v/ v) and blended for 5 min. Organic extract and powder were separated by filtration. The following extractions from recovered powder were carried out by successive volumes of solvant: 2 x 100 ml then 50 ml of redistilled Folch. Final volume of lipid extract was about 400 ml. The organic extract was washed with 150 ml of 0.1 M NaCl solution and dried over anhydrous $MgSO_4$ at 4 °C for 12 h. The extract was filtered and vacuum evaporated to exactly 50 ml. 1 ml of lipid extract was added with 0.5 ml of cholesterol solution in chloroform at 100 µg/ml (internal standard) and dried under a stream of dry nitrogen. For the analysis of total (esterified and unesterified) phytosterols, lipid compounds were saponified by using 0.5 ml of 33 % methanolic KOH in a sand-bath at 65 °C for 1 h. After cooling, sterols were extracted with hexane (5 ml) and trimethylsilyl (TMS) ethers were prepared from only 1 ml of extract with BSTFA (1 % TMCS) as previously described (GAMBERT *et al.* 1979)

C o u p l e d G C - M S a n a l y s i s : A Packard 427 gas chromatograph equipped with a CP-Sil 5 CB capillary column (25 m, 0.32 mm i.d., film thickness 0.12 µm) and a flame ionisation detector was used. TMS derivatives were separated by programming the column temperature from 240 to 290 °C at 1°C/min. The split injector (Ross injector) was held at 260 °C and the detector at 290 °C. Carrier gas flow rate was 1.5 ml/min of He. The methylene units (MU) values of phytosterols were determined by simultaneous injection of C30 and C34 n-alcanes with sample. Relative retention times (RRT) were also calculated using cholesterol as internal standard.

The coupled GC-MS technique used was previously described (MAUME *et al.* 1979). The quadrupole mass spectrometer was a Ribermag R10-10C coupled with a gas chromatograph equipped with a SE30 capillary column. Helium was used as carrier gas. The column temperature was programmed from 220 to 290 °C at 2 °C/min. Ionization was obtained by electron impact : electron energy (70 eV) and filament current (0.19 mA). The temperature of ion source and all connection parts was 250 °C.

Results and discussion

Q u a l i t a t i v e a n a l y s i s o f p h y t o s t e r o l s : Several identification parameters are used. Qualitative analysis is carried out by using 3 standard compounds: campesterol, stigmasterol and ß-sitosterol. Relative retention times/cholesterol and methylene units values of TMS ethers obtained by GC are given in Tab. 1. An example of chromatogram is presented in Fig. 1. From standard compounds and grape skins samples analysis, the datas of mass spectra, molecular ions, base peaks and major fragments (Tab. 2) are in agreement with the results of KNIGHTS (1967), BROOKS *et al.* (1968) and STEEL and HENDERSON (1972). According to GALLANDER and PENG (1976) and FREGA *et al.* (1982), campesterol, stigmasterol and ß-sitosterol are found in grape skins in all analyzed samples. Mass spectrometry analysis also allows to identify lanosterol, the first cyclic intermediate in the biosynthesis of phytosterols in plants, by comparison to data reported in the literature (BROOKS *et al.* 1968; DJERASSI 1978).

O p t i m i z a t i o n o f s t e r o l e x t r a c t i o n : The yield of extraction of 3 sterols (campesterol, stigmasterol and ß-sitosterol) is estimated by using two

T a b l e 1

Identification parameters of phytosterols: Relative retention time (RRT) and methylene unit values. (1) standard compounds (solution in chloroform at 4 µg/ml); (2) RRT towards cholesterol as internal standard ± prediction interval (5 %), n = 20; (3) Methyl ene unit values ± prediction interval (5 %), n = 12

Paramètres d'identification des phytostérols: temps de rétention relatifs (RRT) et unités méthylène. (1) composés standard (solution dans le chloroforme à 4 µg/ml); (2) RRT par rapport au cholestérol (étalon interne) ± intervalle de prédiction (5 %), n = 20; (3) unités méthylène ± intervalle de prédiction (5 %), n = 12

Sterol (1)	RRT (2)	Methylene unit value (3)
Campesterol	1,2087 ± 0,0046	31,809 ± 0,08
Stigmasterol	1,2807 ± 0,0048	32,125 ± 0,111
ß-sitosterol	1,4195 ± 0,0071	32,719 ± 0,112

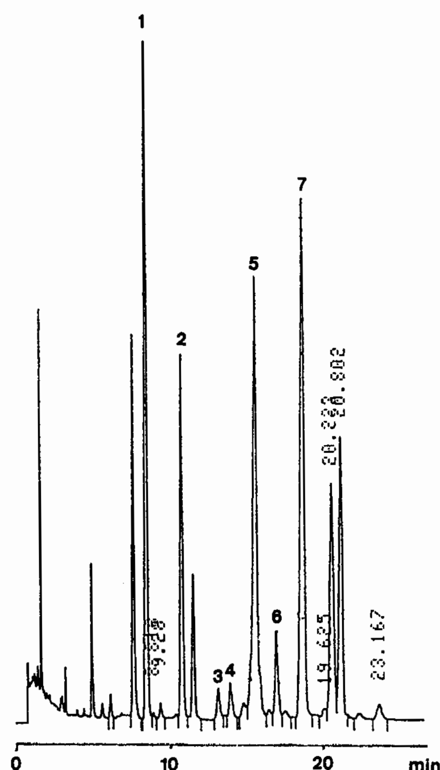

Fig. 1: Analysis by gas chromatography of phytosterols in grape skins. Chromatogram of sample "La Grande Châtelaine" harvested the 10/09/91. Sterols were analyzed as trimethylsilyl ethers by GC with a CP-Sil 5 CB capillary column programmed at 1 °C/min from 240 °C to 290 °C. 1: n-alcane C30; 2: cholesterol (int. standard); 3: campesterol; 4: stigmasterol; 5: ß-sitosterol; 6: lanosterol; 7: n-alcane C34.

Analyse par chromatographie en phase gazeuse des phytostérols dans les pellicules de raisin. Exemple de chromatogramme obtenu pour l'échantillon "La Grande Châtelaine" récolté le 10/09/91. Les stérols sont analysés sous forme d'ethers de triméthylsilyle sur une colonne capillaire CP-Sil 5 CB en programmation de température de 240 °C à 290 °C, à 1 °C/min (1, 2 ... 7: voir la légende anglaise).

Table 2

Mass spectra of phytosterols in grape skins. The sterols were analyzed as trimethylsilyl ethers by GC-MS with a SE30 capillary column programmed at 2 °C/min from 220 to 290 °C. Mass spectra source is used in the electron impact mode at 70 eV. (1) Identification by comparison with standard mass spectrum; (2) identification based on literature related to TMS ether. The number in parentheses means intensity in percent to the base peak.
a $[M-15]^+$; b $[M-90]^+$: $[M-((CH3)3-SiOH)]^+$; c $[M-90-15]^+$; d $[M\text{-side chain}]^+$; e $[M\text{-side chain}-2H]^+$; f [M-side chain-90]+; g $[M\text{-side chain}-90-2H]^+$; h $[M-129]^+$; i $[M-131-2H]^+$; j $[M-90-42]^+$: $[M-90-(C(15) \text{ to } C(17))]^+$; k $[M\text{-side chain}-90-42]^+$; m $[M-131]^+$; n $[M\text{-side chain}-26]^+$: $[M\text{-side chain}-(C(16) \text{ to } C(17))]^+$

Données des spectres de masse des phytostérols analysés dans les pellicules de raisin. Les stérols sont analysés par couplage CPG-SM sous forme d'ethers de triméthylsilyles en utilisant une colonne capillaire à la température programmée à 2 °C/min à partir de 220 °C. L'ionisation est obtenue par impact électronique d'une énergie de 70 eV. (1) identification par comparaison avec le spectre de masse du composé pur; (2) identification basée sur la comparaison avec le spectre de masse de l'ether de TMS donné dans la littérature. Le chiffre entre paranthèses correspond à l'intensité du pic exprimé en pourcentage par rapport au pic de base (voir la légende anglaise).

Identification of sterol	Molecular ion (M+)	Base peak	Major ions in mass spectrum
Campesterol (1)	472 (29)	129	457a (7), 382b (53), 367c (22), 345d (8), 343e (67), 255f (12),253g (2), 217 (7)
Stigmasterol (1)	484 (49)	129	469a (6), 394b (44), 379c (14), 355h (17), 352j (13), 351i (21), 345d (5), 343e (12), 255f (50), 253g (11), 213k (12)
ß-sitosterol (1)	486 (35)	129	471a (9), 396b (67), 381c (25), 357h (73), 355m (7), 345d (3), 343e (2), 329n (4), 255f (15), 217 (9), 213k (11)
Lanosterol (2)	498 (24)	393c	483a (27), 408b (52), 387d (3), 365i (47), 297f (8), 255k (11), 241 (11), 227 (13) 135 (35), 109 (55)

fractional volumes of redistilled Foch mixture. This is carried out on homogeneous sample of skin powder. The subject is to determine the highest efficiency of two extraction methods expressed in µg of each sterol per g of dry weight during successive volumes of solvant. The results (Tab. 3) show that the second method (trial 2) allows to obtain 100 % for both fraction 1/ total extracted campesterol and stigmasterol ratios. For ß-sitosterol, the first and second methods give approximatively the same value. It is

Table 3

Comparison of extraction efficiency between two fractional volumes of solvant (Folch mixture : chloroform-methanol, 2:1 v/v). Sterol content expressed in µg/g of dry weight (skin powder)

Comparaison de l'efficacité de l'extraction entre deux volumes fractionnés de solvant (mélange de Folch : chloroforme-méthanol, 2:1 v/v). Teneur en stérols exprimée en µg/ g de matière sèche (poudre de pellicules)

	TRIAL 1				TRIAL 2			
	fraction 1 150+100+50 mL	fraction 2 50 mL	fraction 3 50 mL	fraction 1/ total	fraction 1 150+100+100+50 mL	fraction 2 50 mL	fraction 3 50 mL	fraction 1/ total
Campesterol	29	5	3	78.4 %	42	-	-	100%
Stigmasterol	71	6	4	87.6 %	88	-	-	100%
ß-sitosterol	950	33	22	94.5 %	968	28	17	95.5 %

clear that the appropriate fractional extraction from 5 g of skin powder is the use of only fraction 1 of second method. These results are completed by reproducibility test of previously described extraction method. It is carried out from the same sample of skin powder. Exactly the same procedure is reproduced 4 times. The variation coefficients are respectively 7.8, 7.1 and 1.0 % for campesterol, stigmasterol and ß-sitosterol. The averages and standard deviations due to extraction protocol calculated ($p < 0.05$) for each sterol are respectively 43.2 ± 5.4, 85.3 ± 9.7 and 969.2 ± 15.2 for campesterol, stigmasterol and ß-sitosterol, expressed in µg/g of dry weight.

Evolution of sterol content in grape skins during the last stage of ripening : During ripening, 4 samplings for Meursault Bouches Chères vineyard and 5 for La Grande Chatelaine vineyard, major phytosterols in grape skins are analyzed by GC. According to FREGA *et al.* (1982), the main sterol compound in skins is generally ß-sitosterol. This result is evidenced in all samples for the last stage of grape ripening (Fig. 2). At the first sampling (September 3), the ß-sitosterol content is about 0.1 % (dry weight basis). The evolution of ß-sitosterol content in grape skins shows a decrease during last stage of ripening. This phenomenon occurs very markedly in "La Grande Châtelaine" sample between September 3 and ripeness. NES (1987) descibes the principal function of sterols mainly found in the plasma membrane (LANGE and RAMOS 1983; RAMGOPAL and BLOCH 1983). He indicates that the principal role of sterols is to act as architectural components. The change of the physical properties of the membrane, especially the rigidity (DAHL *et al.* 1981), can be related to the degrees of ripening in grape skins. According to the study of BAUMAN *et al.* (1977), the neutral lipid fraction decreases before the end of ripening of grapes and markedly increases at peak maturity. This phenomenon is especially illustrated in Meursault vineyard sample. This result can be related to the technological maturity of grapes. At this stage, the berries could be considered as over-maturated. In all samples, ß-sitosterol is about 86 to 89 % of the total analyzed phytosterols. In contrary, campesterol and stigmasterol represent respectively about 5 to 6.5 % and 4 to 7.5 % of total detected phytosterols in grape skins. These results are in agreement with the results of FREGA *et al.* (1982). The evolution of campesterol and stigmasterol in grape skins during ripening is shown in Figs. 3 and 4. It is clear that last time of ripening induces a regular loss in both campesterol and stigmasterol contents. Like with ß-sitosterol, an increase in campesterol and stigmasterol contents occurs at peak maturity.

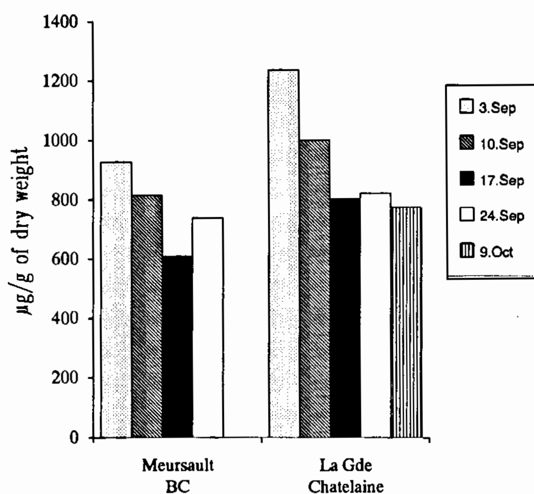

Fig. 2: Evolution of β-sitosterol content in grape skins for the last stage of ripening. Analysis on both "Meursault Bouches Chères" and "La Grande Châtelaine" vineyards.

Evolution de la teneur en ß-sitostérol dans les pellicules de raisin pendant les derniers stades de la maturation. Analyses sur les parcelles de "Meursault Bouches Chères" et "La Grande Châtelaine".

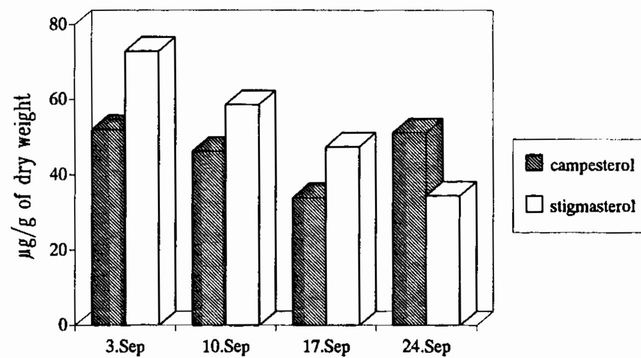

Fig. 3: Evolution of campesterol and stigmasterol contents in grape skins during the last stage of ripening. Meursault Bouches Chères vineyard.

Evolution des teneurs en campestérol et stigmastérol dans les pellicules de raisin au cours des derniers stades de la maturation. Parcelle de Meursault Bouches Chères.

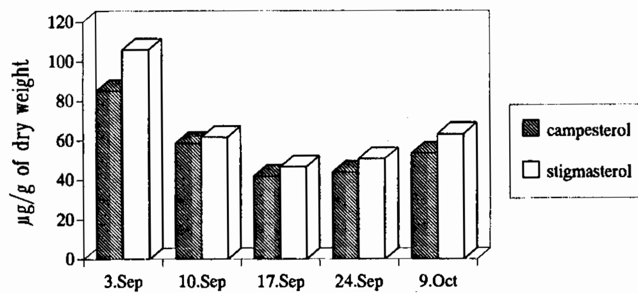

Fig. 4: Evolution of campesterol and stigmasterol contents in grape skins during the last stage of ripening. La Grande Châtelaine vineyard.

Evolution des teneurs en campestérol et stigmastérol dans les pellicules de raisin au cours des derniers stades de la maturation. Parcelle de La Grande Châtelaine.

The relationship between total phytosterols concentration in grape skins and S/A ratio during last weeks of ripening is shown in Fig. 5. The differences between S/A ratio values calculated for two vineyards are due to conditions of maturation. The level of ripening is higher for Meursault 1er cru. It can be explained by over-maturation phenomenon. However, correlation coefficients are deter-

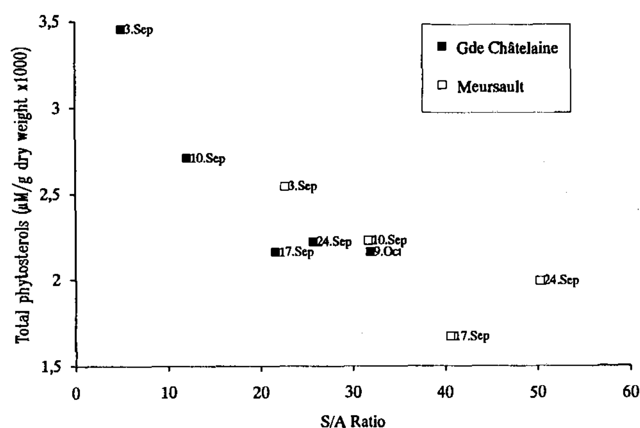

Fig. 5: Relationship between evolutions of both total detected phytosterols expressed in µM/g of dry weight (x 1000) and S/A ratio during the last stage of ripening in grape skins. Analysis carried out on two vineyards: Grande Châtelaine and Meursault 1er cru. S/A: Sugar (g/l)/Total titratable acidity (g/l H_2SO_4).

Relation entre l'évolution de la concentration en phytostérols totaux exprimée en µM/g de poids sec (x 1000) et celle de l'indice S/A au cours des derniers stades de la maturation dans les pellicules de raisin. Analyses effectuées sur les deux vignobles: Grande Châtelaine et Meursault 1er cru. S/A: voir la légende anglaise.

mined for each phytosterol (expressed in mg/g of dry weight) by comparison with S/A ratio from september 3 to 17. Correlation coefficients are respectively - 0.98, - 0.99 and - 0.98 for campesterol, stigmasterol and ß-sitosterol on Meursault 1er cru vineyard. They are respectively - 0.97, - 0.93 and - 0.99 on Grande Châtelaine vineyard. The negative correlation between the sterol concentration in grape skins and S/A ratio may be markedly considered as new index of ripening degree. The results illustrate that during grape ripening, the last phase has a strong influence on the lipid fraction concentration in grape skin.

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