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REVIEW: QUANTITATIVE EXTRACTION AND ANALYSIS OF GRAPE AND WINE PROANTHOCYANIDINS AND STILBENES

REVISÃO: EXTRACÇÃO E ANÁLISE QUANTITATIVA DE PROANTOCIANIDINAS E ESTILBENOS DA UVA E DO VINHO

Baoshan Sun, M. Isabel Spranger

Estação Vitivinícola Nacional, Instituto Nacional de Investigação Agrária e des Pescas, 2565° 191 Dois Portos, Portugal. E-mail: inia.evn.quim@oninet.pt

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SUMMARY

From the point of view of biological importance, proanthocyanidins (condensed tannins) and stilbenes (1,2-diarylethenes) are the two most important classes of polyphenols in grape and wine, which have attracted considerable attention of the international scientific community during the last fifteen years, due essentially to their potential beneficial health effects, related to their protective action towards cardiovascular disease and the oxygen free radical scavenger capacity. Numerous research works on proanthocyanidins and stilbenes have been published, but the majority of them have focused on the structural identification and biological activities of these compounds. Furthermore, less attention has been paid on their quantitative aspects and few analytical methods have been validated. This paper introduces briefly grape and wine proanthocyanidins and stilbenes and presents some works realized in our laboratory concerning quantitative extraction and analysis of these compounds.

Key words: grape, wine, proanthocyanidins, stilbenes, extraction, analysis.

Paravras chave: uva, vinho, proantocianidinas, estibenos, extracção, análise.

INTRODUCTION

The four classes of plant biopolymers – the polynucleotides, proteins, lignins and polysaccharides have been intensively studied over many years and are well recognized. The fifth class of plant biopolymers, *i.e.*, proanthocyanidins, was still not familiar twenty years ago (Porter, 1992) but during the last decade, some spectacular advances in various aspects of scientific research on these phenolic compounds have been achieved. The explosion of research activities on proanthocyanidins over the last years may due to the hypothesis that they are key components responsible for the healthy beneficial effects of

red wine. Stilbenes, another group of phenolic compounds have attracted attention of international scientific committee since the work of Siemann and Creasy (1992). Stilbenes play an important role in both phytopathology and human health. As a consequence, a large number of publications concerning these two groups of phenolic compounds can be found in the literature. However, the majority of these works have focused on the structural identification and biological activities of these compounds. Concerning proanthocyanidins, lower oligomers have long been isolated and well characterized (Porter, 1988; Ricardo-da-Silva *et al*., 1991a), but higher oligomers and polymers, which constitute a large proportion of plant proanthocyanidins (Czochanska *et al*., 1980, Porter *et al*., 1982), were studied and isolated only recently (Souquet *et al*., 1996, 2000; Sun *et al*., 1998a; Guyot *et al*., 1997, 2001). Furthermore, less attention has been paid on their quantitative aspects and few analytical methods have actually been validated. Considering that extensive reviews by experts of this area on various aspects of proanthocyanidins (Porter, 1988, 1994; Rohr, 2002; Kolodziej, 2002) and stilbenes (Cassidy *et al*., 2000; Jeandet *et al*., 2002) in plants have been done very recently, this paper will only give a brief introduction of grape and wine proanthocyanidins and stilbenes and emphasize some works realized in our laboratory on quantitative aspect concerning the extraction and analysis of these compounds.

1. Proanthocyanidins

Proanthocyanidins (condensed tannins) are oligomers and polymers of polyhydroxyflavan-3-ol monomer units linked most commonly by acid-labile 4 → 8 and in same cases by 4 → 6 bonds. 4 → 8 bonds are more common than 4 → 6 bonds and some branching may occur in the chain of higher oligomers and polymers. The term "proanthocyanidins" were thus defined because these colourless compounds release coloured anthocyanidins by cleavage of the interflavan C-C bond on heating in acidic medium. According to their increasing degree of polymerization, proanthocyanidins are termed as follows: dimers, trimers, oligomers and condensed (polymers) (Glories, 1978). The fundamental structural unit of proanthocyanidins is the phenolic flavan-3-ol nucleus (Fig. 1).

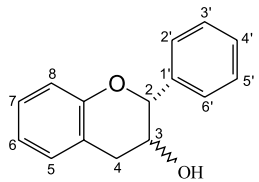


Figure 1. - Chemical structure of flavan-3-ol nucleus.
Estrutura química do núcleo 3-flavanol

The skeleton of flavan-3-ol nucleus can be diversely substituted, in particularly hydroxylated, at the 5- and 7-positions on A-ring, in 3-position on heterocycle and in 3'-, 4'- and/or 5'- on B-ring. The hydroxylation pattern permits to distinguish proanthocyanidins into various classes, including essentially procyanidins, prodelphinidins, propelargonidins profisetinidins and prorobinetinidins (Table I). The first two classes, i.e., procyanidins and

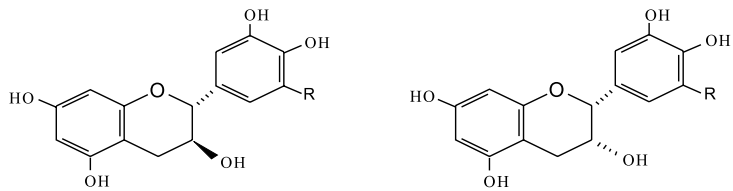
Table I

Major proanthocyanidins encountered in plant tissues

Principais proantocianidinas encontradas no tecidos dos vegetais

Proanthocyanidin class	Constitutive monomer unit	Substitution pattern					
		3	5	7	3'	4'	5'
procyanidin	catechin, epicatechin	OH	OH	OH	OH	OH	H
prodelphinidin	Gallocatechin, epigallocatechin	OH	OH	OH	OH	OH	OH
propelargonidin	Afzelechin	OH	OH	OH	H	OH	H
profisetinidin	Fisetinidol	OH	H	OH	OH	OH	H
prorobinetinidin	Robinetinidol	OH	H	OH	OH	OH	OH

prodelphinidins, are the most commonly encountered in plant tissues. Propelargonidins, profisetinidins and prorobinetinidins are relatively rare. Procyanidins and prodelphinidins release respectively cyanidins and delphinidins when hydrolyzed. Procyanidins are constituted from catechin and epicatechin, whereas prodelphinidins, from gallocatechin and epigallocatechin (Fig. 2).



R = H (+)-catechin R = H (-)-epicatechin

R = OH (+)-gallocatechin **R = OH (-)-epigallocatechin**

Figure 2. - Monomeric units of the major proanthocyanidins

Unidades monoméricas das principais proantocianidinas

The flavan-3-ol unit can be substituted by gallic acid, generally in 3-position to form 3-O-esters of flavan-3-ols. Various flavan-3-ol glycosides have been isolated from plant tissues and the 3-, 5- and 7-O- and 6- and 8-C-glycosides of flavan-3-ols have already been known (Porter, 1988). The structure of proanthocyanidins depends on not only the nature of flavan-3-ol unit but also their polymerization degree and the position linked between these units. Proanthocyanidins can be also divided into two types based on intermonomeric linkages: A-type and B-type. In the case of B-type, the flavan monomers are linked by $C_4 \rightarrow C_8$ or $C_4 \rightarrow C_6$. The A-type proanthocyanidins present one supplementary linkage C_2-O-C_7 or C_2-O-C_5, in addition to linkage $C_4 \rightarrow C_8$ or $C_4 \rightarrow C_6$. Fig. 3 gives examples of B-type and A-type proanthocyanidins.

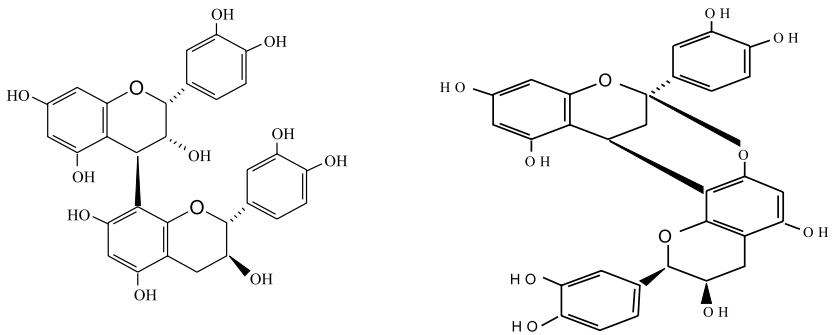


Figure 3. - Examples of B-type and A-type proanthocyanidins.
Exemplos das proantocianidinas do tipo B e tipo A

The greater proportion of proanthocyanidins in plant tissues is often in the form of higher oligomers and polymers. The average polymerization degree of such proanthocyanidins may vary widely. It may be 20-30, as in leaf of *Trifolium affine* (Jones *et al*., 1976), 83 in grape skins (Souquet *et al*., 1996) and 190 units in cider apple (Guyot *et al*., 2001). Fig. 4 demonstrates general structure of proanthocyanidins.

The proanthocyanidins are widely distributed in the plant kingdom: from ferns and their allies to the most advanced di- and mono-cotyledonous plants. In vine, proanthocyanidins are located in leaf (Bourzeix *et al.*, 1986; Boukharta, 1988), in shoot (Boukharta, 1988; Boukharta *et al.*, 1988) and in solid parts of grape cluster (skin, seeds and stems) (Su et Singleton, 1969; Weinges and Piretti, 1971; Piretti *et al.*, 1976; Czochanska *et al.*, 1979; Lea *et al.*, 1979; Haslam, 1980; Bourzeix *et al.*, 1986; Romeyer *et al.*, 1986; Lee and Jaworski,

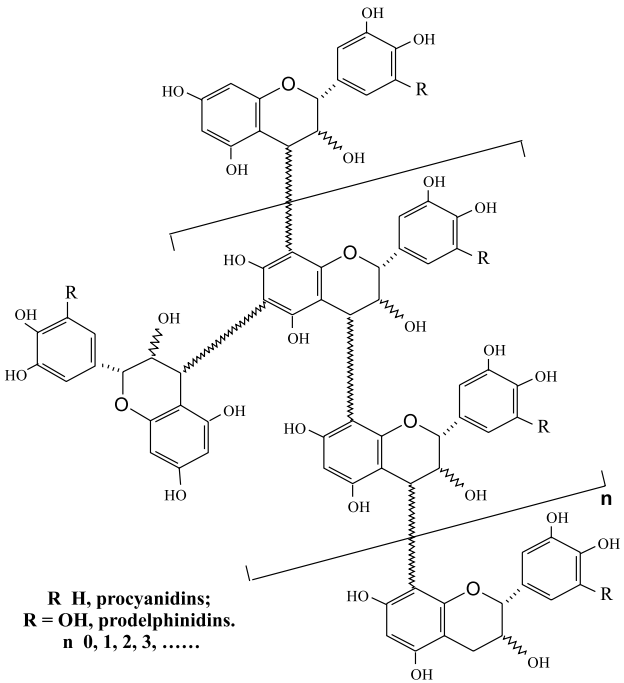


Figure 4. - General structure of proanthocyanidins.

Estrutura geral das proantocianidinas

1987, 1989, 1990; Boukharta *et al.*, 1988; Lunte *et al.*, 1988; Oszmianski and Sapis, 1989, Sun *et al*., 1999a, 2001a), and very little in pulp (Bourzeix *et al.*, 1986; Ricardo-da-Silva *et al*., 1991b, 1992; Sun *et al*., 2001a). The seeds account for a largest proportion of total proanthocyanidins in the entire grape cluster, then the stem and skin; the pulp is free or lack of these compounds (Bourzeix *et al*., 1986; Sun *et al*., 2001a). Proanthocyanidins in seeds are procyanidins composed by catechin, epicatechin and epicatechin 3-O-gallate. In stems, proanthocyanidins are predominantly in form of procyanidins together with low amount of prodelphinidins (Souquet *et al.*, 1998a, 2000; Sun, 1999), while grape skin contained important amount of both procyanidins and prodelphinidins (Souquet et al., 1996, 1998b) in polymeric forms. Wine proanthocyanidins are extracted from solid parts of grapes during fermentation/ maceration. Grape stems contribute to wine both oligomer and polymer proanthocyanidins, grape skins are the important source of polymeric proanthocyanidins to wine, while grape seeds contribute only oligomer procyanidins to wine (Sun *et al*., 1999a). From the quantitative point of view, proanthocyanidins are the major group of polyphenols, both in grape and in wine (Spranger *et al*., 1998). In grapes, catechin and proanthocyanidin levels

vary considerably, depending on the variety (Ricardo-da-Silva *et al.* 1991, 1992; Fuleki and Ricardo-da-Silva, 1997; Sun *et al.*, 2001a), ripening degree (Jordão *et al.*, 2001a,b; Ó-Marques *et al.*, 2005) and the year of harvest (Sun *et al.*, 2001a). On average and on the basis of fresh weight, the concentrations of proanthocyanidins are as follows: total monomers (catechins), 2 - 12 mg/g in seed and 0.1 – 0.7 mg/g in skins; total oligomers, 19 – 43 mg/g in seed and 0.8 – 3.5 mg/g in skins and total polymers, 45 – 78 mg/g in seed and 2 – 21 mg/g in skins ((Sun *et al*., 2001a) For individual procyanidins, in grape seed, the concentration of (+)-catechin is similar to or a little lower than that of (-)-epicatechin, while procyanidin B_2 is predominant among the dimers; in grape skin and stems, the concentration of (+)-catechin is much higher than that of (-)-epicatechin, while procyanidin B_1 is predominant among the dimers, the HPLC profile of which is similar to that of red wine.

The composition of red wine polyphenols is more complex than that of grape. Grape polyphenols mainly consist of catechins, oligomeric and polymeric proanthocyanidins, anthocyanins, phenolic acids, resveratrol and its derivatives, flavonols, flavanonols and flavones. Red wine polyphenols include both grape polyphenols and new phenolic products formed from them during winemaking process and ageing. The enzymatic and non-enzymatic reactions start as soon as the beginning of wine making (crushing) and continue throughout fermentation and ageing. This leads to a great diversity of new polyphenols. As a consequence, wine proanthocyanidins are present both in free form and combined form (*i.e.*, anthocyanin-proanthocyanidin complexes). In one-year-old of young red wines, the concentration of total catechins ranged from 19 to 76 mg/L, oligomers from 65 to 280 mg/L and polymers from 335 to 611 mg/L (Sun *et al*., 2001a). Furthermore, the concentrations of proanthocyanidins in young red wines depend on grape variety and winemaking technology (Sun *et al*., 2001a,b). For the aged red wine the concentrations of all individual phenolic compounds, including proanthocyanidins, were significantly reduced (Sun *et al.*, 2003a, 2005a). The degradation rate is dependent on the nature of phenolic compounds and the winemaking technology used. It has been found very recently that the degradation of anthocyanins was more remarkable than that of proanthocyanidins and proanthocyanidins in carbonic maceration wine appeared more stable than in skin fermentation wines during ageing in bottle (Sun *et al.*, 2005a).

On the other hand, grape and wine proanthocyanidins are presented essentially in polymeric forms (60-80%), followed by oligomeric forms (15-30%), while monomer flavan-3-ols (catechins) represent only a small proportion (less than 10%) (Sun *et al*., 2001a).

Extraction

It seems that there has been not a completely satisfactory or standard method used for quantitative extraction of phenolic compounds from plant tissues, such as grape solid parts, although many extraction techniques have been proposed (Aubert and Poux, 1969; Bourzeix *et al.*, 1975; Darné and Madero-Tamargo, 1979; Bourzeix *et al.*, 1986; Tiarks *et al.*, 1992). Furthermore, quantitative extraction of phenolic compounds from plant tissues has been a constant challenge to chemists due to various interfering parameters involved - particle size of the sample, type (or nature) of the solvent(s), time and temperature of extraction, pH of the extraction medium, number of extraction, degradation of compounds during extraction, *etc* (Deshpande *et al.*, 1986). Among these factors, the type of extraction solvent used is the most important because it can affect the amount extracted, the biological activity, the molecular mass and the type of phenolic compounds (Deshpande *et al.*, 1986; Tiarks *et al.*, 1992). The most used extraction solvents for proanthocyanidins were methanol, methanol-water, and acetone-water. They might be single-used (Thompson *et al.*, 1972; Scalbert and Haslam, 1987; Oszmianski and Lee, 1990) or combined (Tiarks *et al.*, 1992). Sometimes, ethanol or ethyl acetate was also used (Asquith and Butler, 1985; Yokotsuka and Singleton, 1987). However, the proanthocyanidins quantified were only those extracted by the solvent used. Since proanthocyanidins in different plant tissues are presented in different binding states, it is impossible to have one optimized extraction method for all types of plant tissues. In other words, we should keep in mind that, one optimized quantitative extraction method would indicate that (1) it permits "*maximum*" extraction of the desired phenolic compounds and (2) the method is reproducible and the results obtained from same or different tissues can be meaningfully compared. In this part, we will emphasize the solvents used for extraction of proanthocyanidins from grape solids. However, other factors are also important for both qualitative and quantitative extraction purpose, which is summarized as follows: (1) *pH of the extraction medium*. Acidified solvent(s) have been often used for extraction of anthocyanins (Somers, 1968) and phenolic acids (Xu and Diosady, 1997; Metivier *et al*., 1980), but it is not advisable, according to our experiences, for extraction of proanthocyanidins from solid parts of grape. This is because the usual solvents as acetone or methanol are sufficiently strong while the use of acidified solvent(s) may induce degradation of proanthocyanidins during extraction. Moreover, acids present in the final extracts are difficult to eliminate. Since the final extracts are generally needed to be concentrated or evaporated prior to analysis by other methods, the acidity of the concentrated solution would be significantly increased and thus run a risk to hydrolyze proanthocyanidins; (2) *Particle size*. To ensure high extraction efficiency, solid materials should be ground finely. Grinding or homogenizing is often performed in the presence

of extraction solvent. Ball grinder and high-speed dry miller are generally used; (3) *Temperature.* On one hand, increasing temperature increases the solubility and diffusion of the compounds to be extracted, decreases the viscosity of solvent and thus enhances extraction efficiency. On the other hand, high temperature may degrade proanthocyanidins. From quantitative and practical point of view, it is preferable that extraction is performed at room temperature; (4). *Oxidation or degradation.* Proanthocyanidins are easily oxidized or degraded, so precaution should be paid throughout the extraction procedure. In addition to high temperature as described above, light and air should be also avoided. Thus, fresh grape solids (seed, stem and skin), once isolated, should be immediately, or stored at –20ºC, or lyophilized, or macerated in extraction solvent followed by extraction procedure. Extraction should be conducted in the presence of nitrogen gas under darkness. Although several authors reported that addition of ascorbic acid to extraction solvent can increase stability of proanthocyanidins (Prieur *et al*., 1994; Nitao *et al*., 2001), ascorbic acid may also induce browning of catechin (Bradshaw *et al*., 2001) and thus limit its use.

In grape cluster, grape seeds are a part the richest in proanthocyanidins but a part where proanthocyanidins are the most difficult to extract. Fig. 5 presents our previous work concerning the extraction efficiencies of different solvents on grape seed proanthocyanidins (Sun et al., 1996).

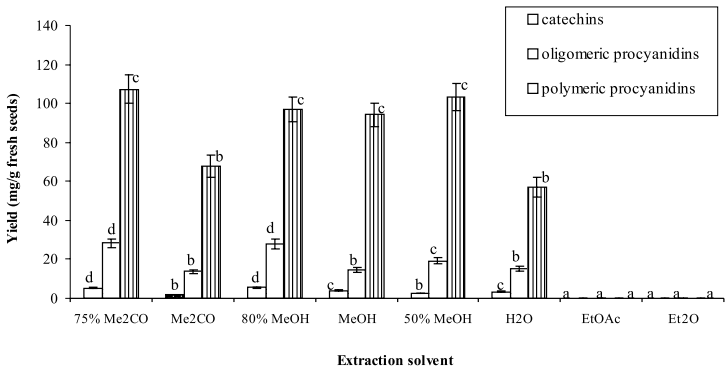


Figure 5. - Extraction efficiency of various solvents on grape seed procyanidins. Me_2CO = acetone; MeOH = methanol; EtOAc= ethyl acetate; Et_2O = diethyl ether. Vertical bars represent the standard deviation (n = 4). For the same fraction, means followed by the same letter are not significantly different (LSD, 99%). Adapted from Sun et al. (1996).

Eficiência de Extracção de varios solventes para as procianidinas das graínhas de uva. Me_2CO = acetona; MeOH = metanol; EtOAc= acetato de etilo; Et_2O = éter dietílico;. barras verticais representam o desvio padrão (n = 4). Para a mesma fracção, as médias seguidas por a mesma letra indicam que não há diferença significativa (99%, LSD). Adaptado de Sun et al. (1996).

For each solvent, three sequential extractions appeared sufficient; the first extract contains over 90% of total extractable proanthocyanidins while only trace amount of these compounds were found in the third extract. It should be especially mentioned that any tested solvent (with three sequential extractions) gave very good repeatability, but this does not signify that the three sequential extractions using each solvent could be considered as one quantitative extraction method, because the yields of procyanidins obtained by different solvents are considerably different. For grape seeds, neither ethyl acetate nor diethyl ether could be used as extraction solvent both for quantitative and preparative purposes. In fact, ethyl acetate extracted catechins and oligomeric procyanidins in a small amount, and diethyl ether extracted only catechins, also in a little quantity. Water may be an interesting solvent for preparation of procyanidin products for food or pharmaceutical industry because it is non-toxic and gives an acceptable yield of procyanidins (Fig. 5). However, water alone can extract and dissolve a large amount of protein and polysaccharide which will cause problems for further analysis of procyanidins. Figure 5 showed clearly that 80% methanol and 75% acetone were the best extraction solvents both for catechins and oligomeric and polymeric procyanidins from grape seeds. Methanol and 50% methanol were also good extraction solvents for seed polymeric procyanidins although they gave less extraction efficiencies for catechins and oligomeric procyanidins than 80% methanol and 75% acetone. These results may partially explain why single-solvent methanol, methanol° water or acetone-water is found in the literature to be the most used for qualitative or quantitative extraction of plant polyphenols. However, we should keep in mind that a given single-solvent only extract one type or one binding state of polyphenols (or proanthocyanidins). Amrani Joutei *et al.* (1994) studied the localization of proanthocyanidins in grape skins and in grape seeds. It was found that there were three groups of proanthocyanidins present in the skin tissues: the first group consisted of free proanthocyanidins in solution in the vacuolar sap and appeared in the form of granules decreasing in size from the skin surface to the pulp; the second group of proanthocyanidins were bound to the proteins of the internal face of the tonoplast, whereas the third group of proanthocyanidins were bound to the cell wall polysaccharides by osidic bonds. In grape seeds, these authors found that procyanidins were located both in outer coat and in the endosperm. Moreover, Thorngate and Singleton (1994) reported that the grape seed procyanidins were essentially localized in the outer coat, with the endosperm being much lower in these compounds.

Furthermore, when extraction is complete by one solvent, the use of another solvent of different nature would extract more polyphenols. This is why the method proposed by Bourzeix *et al.* (1986) has been used as a standard method for quantitative extraction of grape proanthocyanidins over years in our laboratory (Sun *et al*., 1998a, 1999a, 2001a) and by other authors (Ricardo°

da-Silva *et al*., 1991b, 1992) The advantage of this method is the combination of several solvents of different polarities, which permits more complete extraction of proanthocyanidins from different parts of grapes. This method is described as follows. The extraction is performed under nitrogen gas and in darkness. Sequential extraction was performed in following order: methanol during one night at -20ºC, 80% methanol in water during 4 hours at room temperature, 50% methanol in water during 4 hours at room temperature, distilled water for freezing at – 20 ºC during 15 hours and finally, 75% acetone in water during one hour at room temperature. This method is reproducible and the values obtained from different parts of grape cluster can be meaningfully compared (Sun *et al*., 2001a). The disadvantage of this method is that it is very time and solvent consuming. Since 80% methanol and 75% acetone were the best solvents for extraction of procyanidins from grape seeds as mentioned above (Fig. 5), we proposed an alterative extraction procedure, for preparation purposes, by using only these two solvents: 80% methanol followed by 75% acetone (Sun *et al*., 1999b). The extraction efficiency of the proposed method for grape seed proanthocyanidins, as compared with that of Bourzeix *et al.* (1986), is verified and presented in Table II.

Table II

Comparison of the two different methods for extraction of proanthocyanidins from grape seeds

Comparação de diferentes métodos para a extracção das proantocianidinas de graínha da uva

Sample	Proanthocyanidins	Amount extracted (mg/g dry weight)		Extraction efficiency of the proposed preparation method (%)*
		using the proposed preparation method	using the method of Bourzeix et al (1986)	
Sd1	Monomer (catechins)	5.35 ± 0.45	5.58 ± 0.10	100
	Oligomer	27.50 ± 2.17	43.90 ± 2.18	62.6
	Polymer	85.94 ± 0.80	154.86 ± 6.78	55.5
Sd2	Monomer (catechins)	6.25 ± 1.36	6.16 ± 0.90	100
	Oligomer	22.32 ± 1.50	33.56 ± 3.78	66.5
	Polymer	118.06 ± 13.55	159.19 ± 0.95	74.2

* Extraction efficiency of the proposed preparation method = (amount extracted using the proposed preparation method / amount extracted by he method of Bourzeix et al) × 100%.
Sd1 = Grape seed of Fernão Pires variety harvested in 1997; Sd2 = Grape seed of Fernão Pires variety harvested in 1999.

It can be seen, from table II, that there is no significant difference in total amount of monomer proanthocyanidins (catechins) extracted by the two methods, indicating that the proposed method by us are very efficient for extraction of catechins from grape seeds. However, the method we proposed

extracted less amounts of oligomer and polymer proanthocyanidins than those by the method of Bourzeix et al. (1986). The reason for this may be explained by the fact that the two solvents 50% methanol and water used by Bourzeix *et al.* (1986) permit extract also highly polar proanthocyanidins. It should be emphasized that this method we proposed is much less time and solvent consuming and thus more economical and practical. In addition, although the yield of procyanidins extracted by combination of the two solvents was lower than that obtained by the method of Bourzeix *et al*. (1986) it is significantly higher than any of their single solvent (Sun *et al.*, unpublished data). As a consequence, the method of Bourzeix *et al*. (1986) is proposed to be used as quantitative extraction method, while the sequential extraction by 80% methanol and 75% acetone we proposed is used for preparation purposes (Sun *et al.* 1999b). In a word, for extraction of polyphenols in plants, the choice of extraction solvent depends on the structure of plant tissue and the binding state and type of polyphenols, and also depends on whether the objective is quantitative or qualitative. For example, for monitoring the variation of proanthocyanidins in grape skins during maturation, extraction with strong single solvent such as 80% methanol or 75% acetone may be valid for parallel comparison purposes. Combination of this sequential extraction with recently proposed microwave-assisted extraction technique (Csiktusnàdi Kiss *et al*., 2000) would reduce significantly the extraction time.

Fractionation and isolation

Various methods have been proposed to fractionate and isolate proanthocyanidins, among which column chromatography on Sephadex LH° 20 (Lea and Timberlake, 1974; Boukharta *et al*., 1988), on Toyopearl TSK HW-40 (F) (Fulcrand *et al*., 1999; Sun *et al*., 1999b), on Toyopearl TSK HW° 40 (S) (Ricardo-da-Silva, 1991a; Saint-Cricq de Gaulejac *et al*., 1998; De Freitas et al., 1998), on Toyopearl TSK HW-50 (F) (Meirelles *et al*., 1992; Vidal *et al*., 2002) and on LiChroprep RP-18 (Sun et al., 1994, 1999b), solid° phase extraction on C18 Sep-Pak cartridges (Jaworski and Lee, 1987; Oszmianski *et al*., 1988; Revilla *et al*., 1991; Sun *et al*., 1998a; Vidal *et al*., 2002) and liquid-liquid extraction with ethyl acetate (Glories, 1978; Saucier *et al*., 2001), are the most often used. Column chromatography on Toyopearl was also used for fractionation of grape seed proanthocyanidins according to their structural features and to their degree of polymerization (De Freitas et al., 1998). Furthermore, these column chromatography techniques were often used in semi-preparative or preparative work, or for qualitative purposes. From the analytical point of view, they are relatively delicate and time° consuming. Liquid-liquid extraction with ether to isolate monomeric flavanols and with ethyl acetate to isolate oligomeric proanthocyanidins was used long time ago (Geissman and Dittmar, 1965; Stonestreet, 1965) and still is used

today (Saucier *et al*., 2001) for various objectives. However, the recovery is poor. Only partial dimeric procyandins in red wine were confirmed to be extracted by three times of extraction with ethyl acetate (Sun *et al*, unpublished data). In addition, ethyl acetate also extracts a small amount of anthocyanins which might interfere in further analysis of proanthocyanidins.

Solid-phase extraction (SPE) on Sep-Pak cartridges is a rapid and easy method which permits quantitative separation of polyphenols into various classes (Jaworski and Lee, 1987; Oszmianski *et al*., 1988; Revilla *et al*., 1991; Sun *et al*., 1998a), with significant reduction of solvent used. SPE on Sep-Pak cartridges can also be used for quantitative fractionation of grape and wine proanthocyanidins according to their degree of polymerization; successive elution first with ethyl acetate and then with methanol allow to separate monomers and oligomers from polymers; diethyl ether permits to elute monomers while the rest proanthocyanidins can be recovered by methanol (Sun *et al*., 1998a).

Although important progress has been achieved in the development of methods to fractionate proanthocyanidins and to separate them from other phenolic compounds presented in grape skins and red wines, the major problems actually remained is the difficulty to separate oligomeric proanthocyanidins from anthocyanins (Vidal *et al*., 2002) and polymeric proanthocyanidins from pigmented complexes. It appeared that any individual technique mentioned above could not resolve all these problems. For this reason, we have combined several techniques to quantitatively separate red wine polyphenols into various fractions: phenolic acids, free monomeric flavanols, free oligomeric proanthocyanidins, free anthocyanins and their pyruvic derivatives, free and/ or non-pigmented polymeric proanthocyanidins, pigmented proanthocyanidins and other pigmented complexes. The fractionation procedure is presented in Fig. 6.

Not as liquid-liquid extraction, using mini-column C18 only trace amount of anthocyanins was co-eluted together with monomer and oligomer proanthocyanidins (Fraction 2). Moreover, the majority of the co-eluted anthocyanins are presented in small amount of residual water, which can be easily eliminated by freezing the fraction overnight. Using this fractionation procedure, we have, for the first time, isolated free and/or non-pigmented polymeric proanthocyanidins from one-year-old red wine. High purity in proanthocyanidins of this fraction was verified by Thiolysis-HPLC. No anthocyanins were released in the thiolysed solution. Furthermore, the proposed fractionation procedure may be used for quantitative purposes. Since the major groups of polyphenols in red wine can actually be separated one from another, quantification of total amount of specific phenolic compounds in corresponding fraction can thus be carried out by colormetric measurement.

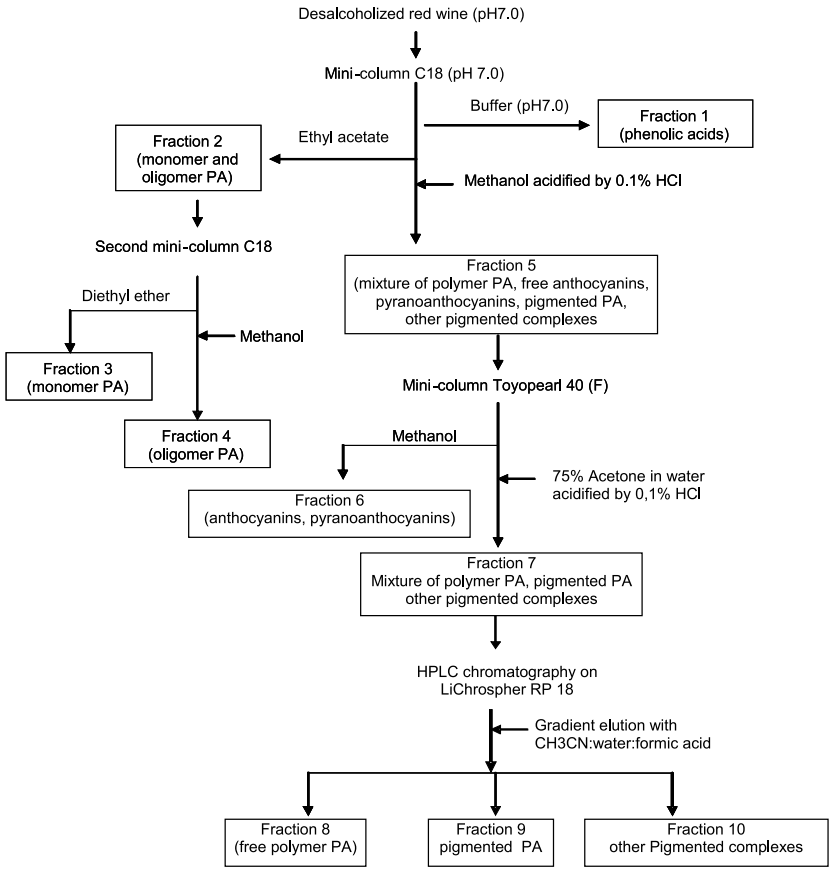


Figure 6. - Fractionation of phenolic compounds in red wine (adapted from Sun et al., 2005b).
PA = proanthocyanidins; Ant = anthocyanidins

Fraccionamento dos compostos fenólicos no vinho tinto (adaptado de Sun et al., 2005b).
PA = proantocianidinas; Ant = antocianidinas

Quantification

Direct quantification or analysis of proanthocyanidins in crude grape phenolic extract or in red wine are often impossible because of the complex sample matrix and the diversity of their proper chemical structures. Thus, fractionation or pre-purification of such samples prior to analysis is often necessary.

Quantification of total amount of proanthocyanidins. Although the methods of Folin-Ciocalteu reagent and Prussian Blue assays are widely used for quantification of total polyphenols or tannins in plants and plant-derived foods,

they are not specific for proanthocyanidins because other phenolic compounds can also react with these reagents. Thus, Vanillin assay and dimethylaminocinnamaldehyde (DMACA) assay are the two colorimetric methods mostly used for quantification of total proanthocyanidins because of their sensitivity, specificity and simplicity, though several factors can affect their precision and accuracy for which precaution should be paid (Sun *et al*., 1998b). They are quite specific to a narrow range of monomer and polymer proanthocyanidins. However, the reactivity of monomer (catechin) to vanillin or to DMACA is much higher at strong acidic medium than that of proanthocyanidins, so using catechin as reference standard, proanthocyanidins are markedly underestimated. For estimating accurately monomer and polymer proanthocyanidins that exist simultaneously in a sample, it is advisable to preliminarily separate them from each other. The separation of catechins from proanthocyanidins in grape and wine can be achieved by SPE on Sep-Pak cartridges C18 as described in our previous work (Sun *et al.*, 1998a) or by mini-column chromatography on LiChroprep RP-18 (Sun et al., 2005b). Catechin and proanthocyanidin fractions should be separately quantified using catechin and the purified proanthocyanidins of the same origin as reference standard, respectively (Sun *et al*., 1998b)

Quantification of individual proanthocyanidins. Proanthocyanidins in grape and wine show a great structural diversity and complexity as compared with those in other plants or plant-derived foods such as apple (Guyot *et al*., 2001), pear (Ferreira *et al*., 2002) and cocoa (Rigaud *et al*., 1993; Hammerstone *et al*., 1999). The number of isomers of proanthocyanidins increases markedly as increases their degree of polymerization (DP). Considering only molecules constituted by catechin and epicatechin, the number of asymmetric carbon atoms is $2^{(3\times DP-1)}$, the number of possible interflavan bonds ($C_4 - C_8$ or $C_4 - C_6$) is $2^{(DP-1)}$ which is also equal to that of possible forms of rotation around these bonds. Thus, theoretically, the possible number of stereo- and rotational isomers is 128 for dimer, 4096 for trimer, and $2^{(3\times DP-1)} \times 2^{(DP-1)} \times 2^{(DP-1)} = 2^{(5\times DP-3)}$ for higher oligomer and polymer,. Although the number of isomers existed in natural plants is much limited due to steric interference, it is an undoubted fact that as the DP of plant proanthocyanidins increases the number of isomers increases dramatically. The number of isomers of grape skin, grape stem and red wine proanthocyanidins is, theoretically, higher than that of grape seed because these tissues contain simultaneously procyanidins and prodelphinidins. As a consequence, the concentration of each isomer for a given highly polymerized proanthocyanidin would be very low. Separation and quantification of individual proanthocyanidins are thus very difficult as compared with those of other phenolic compounds.

Reverse-phase HPLC has been the primary method for quantitative analysis of individual proanthocyanidins in plant and plant-derived food samples.

However, reverse-phase HPLC only permits to separate proanthocyanidins until trimers, while higher oligomers and polymers are co-eluted as a large unresolved peak (Rigaud *et al*., 1993). Since monomers, dimers and trimers make up only a small proportion of total proanthocyanidins, pre-purification of the sample before RP-HPLC analysis is often necessary. The presence of higher oligomer and polymer proanthocyanidins and also other phenolic compounds results in poor resolution and gradual baseline, which makes difficult to quantify accurately the low-molecular-mass compounds. For analysis of di- and trimer procyanidins in grape and wine samples, pre-purification using mini-column on polyamide proposed by Ricardo-da-Silva *et al*. (1990) appeared very useful. Other pre-purification methods as SPE on C18 or on Toyopearl 40 (F) can also be used for this purpose, but appeared less effective. The column used for HPLC is generally reverse-phase C18 and elution is performed by a gradient program using acetonitril-water as mobile phase. Slowing the gradient of acetonitril or even using acidified water as elution solvent (Ricardo-da-Silva *et al*., 1990) improves the resolution.

Normal-phase HPLC has been increasingly used, during the last few years, for separation of procyanidins from cacao (Rigaud *et al*., 1993; Hammerstone *et al*., 1999), grape seed (Rigaud *et al*., 1993; Sun *et al*., 1999c), cider apple (Guyot *et al*., 2001), according to their degree of polymerization. Recently, Hammerstone *et al*. (1999) have successfully separated cocoa and chocolate procyanidins from dimers up to decamers. Furthermore, the same authors and colleagues have also used this method to quantify proanthocyanidins in other food samples (Lazarus *et al*.,1999). However, for separation of grape and wine proanthocyanidins, resolution by normal-phase HPLC is much poorer than that by reverse-phase HPLC and overlapping becomes more important as molecular mass increases.

Depolymerization in the presence of acid and nucleophile followed by HPLC analysis is a useful tool for quantitation and characterization of proanthocyanidins. This method allows determining the nature and concentration of terminal and extension units and consequently calculating the mean DP (mDP) and the percentage of galloylation (%G) of proanthocyanidins. The most used nucleophiles are toluene--α-thiol (benzyl mercaptan) and phloroglucinol. Some authors preferred phloroglucinol to toluene--α-thiol because it is odorless, while toluene--α-thiol gives an unpleasant odor and is toxic. However, the advantage of toluene--α-thiol over phloroglucinol is that it gives much higher yields (Matthews *et al*., 1997) and thus, from analytical point of view, toluene--α-thiol is preferred. Another advantage of toluene--α-thiol is that it could be used for identification purposes, by partial degradation of proanthocyanidins and then reduction with Raney Nickel (Rigaud *et al.*, 1991). The procedure of degradation of proanthocyanidins in the presence of toluene--α-thiol proposed by Prieur *et al*. (1994) appears simple and rapid,

which have been successfully applied, over several years in our laboratory, for determination of structural composition, mDP and %G of grape and wine proanthocyanidins. Furthermore, to ensure a good repeatability of this method, some precaution should be paid and some slight modification of the manipulation was done in our laboratory as follows. The volume of the sample and toluene--α-thiol were increased from 20 to 50 µl; the concentration of proanthocyanidins for highly polymerized fraction should be not less than 3 g/dm^3 (in methanol) due to the relatively low concentration of terminal units of these molecules. After exact 2 minutes of thiolysis reaction at 90ºC, the ampoule is immediately cooled in ice-bath before opened and injected to HPLC, thus permits minimizing side reactions as epimerization of terminal units and avoiding methanol evaporation. Since the injection solvent is methanol, the volume of injection should be reduced (in our case, 10 µl) in order to avoid methanol "effect" which induces shoulder and/or gradual baseline just before the main peaks corresponding terminal units appear.

Electrospray ionization mass spectrometry (ESI-MS) has been proved to be a very powerful tool for characterization of proanthocyanidins, in particular for detection of individual oligomeric and polymeric proanthocyanidins in a mixture or in heterogeneous solutions, providing the molecular mass, number of constitutive moieties and substituents (Guyot *et al*., 1997; Le Roux *et al*., 1998; Hayasaka *et al*., 2003). The advantage of eletrospray ionization is that it permits the detection of the molecular ion but does not cause the fragmentation of the molecule as occurs other types of ionization such as atmospheric pressure chemical ionization (APCI) (De Pascual-Teresa and Rivas-Gonzalo, 2003). Earlier works (Cheynier *et al*., 1997; Fulcrand *et al*., 1999) showed that ESI-MS analysis of grape seed extract detected various procyanidins from dimer (PC2) to pentamer (PC5) as mono-charged ion species ($[M-H]^-$) and from pentamer to nonamer (PC9) as doubly charged ion species ($[M-2H]^{2-}$), with galloylation degree up to 3. Lately, tridecamer (PC13) and its monogallate were detected in an oligomeric procyanidin fraction from grape seeds by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) (Pianet *et al*., 2002). More recently, Hayasaka *et al*. (2003) detected various single ($[M-H]^-$), double ($[M-2H]^{2-}$)and triple ($[M-3H]^{3-}$) charged ions in grape seed fractions corresponding the molecular sizes of procyanidins up to 28 units and with a galloylation degree ranging from 0 to 8, using ESI-MS under enhanced resolution. In our laboratory, ESI° MS was used for detection of highly polymerized proanthocyanidins in grape seed and in red wine. For this purpose, both grape seed extract and red wine were fractionated, in order to obtain purified polymeric proanthocyanidin fractions. The fractions were lyophilized and powder obtained was dissolved in pure methanol to give a concentration of 100 mg/dm^3, prior to MS analysis. Each solution was infused directly into ESI source with a syringe pump (74900

Series, Cole-Parmer Instrument) at a constant flow rate of 180 µl/h. Mass spectra were recorded from m/z 200-3000 in a negative mode, Mass range mode was selected as Standard/Normal, Standard/Enhanced and Standard/Maximum, respectively. Other MS analysis conditions are as follows: capillary voltage –3500 V, nebulizer gas (N_2) 10 (arbitrary units), drying gas (N_2) temperature 350ºC. Multiply charged ions (from $[M-2H]^{2-}$ to $[M-6H]^{6-}$) were identified by deconvoluting high charge states up to 6. Multiple fragmentation experiments MS^n were performed for getting more detailed structural information of target compounds (procyanidins). Various pseudomolecular ions corresponding polymeric proanthocyanidins were thus detected, some of which corresponding grape seed procyanidins are presented in Table III.

Table III

Ions of procyanidins identified by ESI-MS analysis of grape seed polymeric fractions*

*Iões de procianidinas identificadas por análise ESI-MS da fracção polímero de graínha da uva**

DP	Galloyl	$[M-H]^{-1}$	$[M-2H]^{-2}$	$[M-3H]^{-3}$	$[M-4H]^{-4}$	$[M-5H]^{-5}$	$[M-6H]^{-6}$
11	2		1735.9	1157.2	868.0		
11	3		1811.7				
11	8		2191.7				
11	9		2268.1				
12	0				864.4		
12	1		1804.1		902.1		
12	5		2108.3	1404.9			
12	7		2295.9				
13	0	1247.5					
13	1			1298.2			
13	3			1399.8			
13	4			1450.5			
13	5			1501.1			
13	8			1652.9			
13	9			1703.9			
13	11			1805.1			
14	3			1400.5	1121.4		
14	4			1546.4			
14	5			1597.3			
14	10			1850.0			
15	0			1439.6			
15	2					924.5	
15	5					1692.7	
15	6			1839.2			
15	9			1844.3			
16	3			1687.8			
16	7			1890.3			
16	8			1941.1			
16	10			2042.7			
16	11			2092.8			
17	8			2037.2			
18	10			2234.8			

Unpublished data; ions of procyanidins with DP ≤ 10 are not listed in this table.

Furthermore, some ions corresponding proanthocyanidins with DP ≤ 10 (not listed in table III) were also detected in grape seed polymeric fraction, indicating that there was still some overlapping between this fraction and the oligomer fraction. The detected pseudomolecular ions in grape seed oligomeric fraction correspond procyanidins from DP2 to DP18 (data not shown). In addition, there were also many other ions detected in polymeric fraction which would be interpreted as proanthocyanidins with DP ranging from 19 to 32, but their intensities were too low to perform further MS^n analysis. Further work will be done to increase the intensities of these ions.

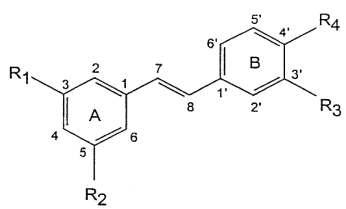
In conclusion, solvents of different natures have very different extraction efficiencies on grape proanthocyanidins. The combined solvent system proposed by Bourzeix *et al*. (1986) has higher extraction efficiency than any single solvent and thus it is recommended as a standard method for quantitative extraction of proanthocyanidins from solid parts of grape cluster. The method of sequential extraction with 80% methanol followed by 75% acetone are less efficient but much less time and solvent consuming and thus can be used for qualitative extraction or for the purposes of preparation of low-molecular proanthocyanidins. For analysis of individual procyanidins (dimers and trimers) from grape extract and wine, fractionation and isolation of oligomer fraction is necessary prior to HPLC analysis, for which mini-column chromatography on polyamide (Ricardo-da-Silva *et al*., 1990) is preferred and recommended. Both vanillin assay and DMACA can be used for quantification of total proanthocyanidins in grape extract and red wine samples, but some precaution should be paid and it is particularly important that monomer flavan-3-ols (catechins) should be preliminarily separated from proanthocyanidins, if they co-exist in the same sample, using the simple method already described (Sun *et al*., 1998a). Thiolysis is a very useful technique for structural characterization of grape and wine proanthocyandins, giving access to obtain mDP, percentage of galloylation and relative percentage of each constitutive unit. Both toluene--α-thiol and phloroglucinol can be used as nucleophilic agent, with the former preferred from analytical point of view, but the latter appeared to be increasingly used recently, due probably to its odorless. Once again, it is necessary or preferable that catechins are preliminarily separated from proanthocyanidins, if they co-exist in the same sample, before thiolysis. ESI-MS has proved to be a very powerful tool for structural assignation of highly polymerized polyphenols. Various polymeric proanthocyanidins from grape seeds (Table III) and in wine (Sun *et al.*, 2005b) have been thus detected. However, the complexity and diversity of highly polymerized polyphenols make often difficulty in ESI-MS or LC-ESI-MS analysis of grape and wine sample by direct injection, due to weak or overlapping signals contributed by numerous mono- and multiply charged ions. Pre-fractionation of grape and wine polyphenols before MS analysis, would improve LC-ESI-MS method. The

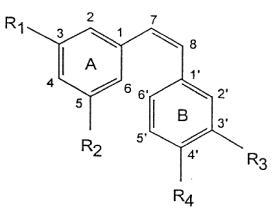
combined chromatography techniques as described in Figure 6 permit to quantitatively separate red wine polyphenols into various fractions, in particular that of free polymeric proanthocyanidins from anthocyanin-derived compounds.

2. Stibenes

Stilbenes (1,2-diarylethenes) belong to non-flavonoid class of phenolic compounds. Their occurrence in plant tissues is associated to the resistance of plant against fungal diseases such as *Botrytis cinerea*, although they can also occur to abiotic stress, such as UV irradiation. Generally, stilbenes are considered as phytoalexins, and their formation in grape leaves has been correlated with disease resistance (Langcake and McCarthy, 1979; Langcake, 1981). Phytoalexins are a group of plant chemicals of low molecular mass which are inhibitory to microorganisms and their accumulation in plants is initiated by interaction of the plant with microorganisms.

Stilbenes can exist as *cis* and *trans* isomers. The major stilbene is its monomeric form - resveratrol (3,5,4'-trihydroxystilbene). This is why the majority of published works over many years have focused on this compound (Jeandet *et al*., 1995a,b; Adrian *et al*., 1996; Bavaresco *et al*., 2001; Bessis *et al*., 1997; Blond *et al*., 1995). However, the presence of other derivatives or glucosylated forms is also well known. Fig. 7 presents the monomeric estilbenes identified in *Vitis genus*.





	R1	R2	R3	R4		R1	R2	R3	R4
1	OH	OH	H	OH	6	OH	OH	H	OH
2	GlcO	OH	OH	OH	7	GlcO	OH	OH	OH
3	OH	OH	H	GlcO	8	OH	OH	H	GlcO
4	GlcO	OH	H	OH	9	GlcO	OH	H	OH
5	MeO	MeO	H	OH	10	MeO	MeO	H	OH

Figure 7. - Chemical structures of monomeric stilbenes identified in *Vitis* genus*:* 1 and 6: *trans*- and *cis*-resveratrol; 2 and 7: *trans*- and *cis*-astringin; 3 and 8: *trans*- and *cis*° resveratroloside; 4 and 9: *trans*- and *cis*-piceid; 5 and 10: *trans*- and *cis*-pterostilbene.

Estructuras químicas de estilbenos identificados no género Vitis: 1 and 6: trans- e cis° resveratrol; 2 e 7: trans- e cis-astringina; 3 e 8: trans- and cis- resveratrolósido; 4 e 9: trans- and cis-"piceid"; 5 e 10: trans- e cis-pterostilbeno.

Stilbenes can be also found to be presented in oligomeric and polymeric forms, so-called viniferins (Bessis *et al*., 1998). ε-viniferin (dimeric resveratrol), α-viniferin (trimeric resveratrol), as well as stilbenoids like ampelopsin A (dimeric resveratrol) and hopeaphenol (dimeric ampelopsin A) have been identified (Jeandet *et al*., 2002).

Stilbenes are present in both edible and non-edible plant tissues. Grape and red wine are among the major dietary sources of stilbenes. However, stilbene concentrations in both grape and in wine are relatively low as compared with other phenolic compounds. In grape cluster, stilbenes are located essentially in skins and mainly in glucosylated form (Creasy and Coffee, 1988, Roggero and Garcia-Parrilla, 1995). These compounds were also reported to be present in grape seeds (Pezet and Cuenat, 1996, Sun *et al.*, 2005c) and grape stems (Bavaresco *et al.*, 1997, Püssa et al., 2005, Sun *et al.*, 2005c). Resveratrol ° the major stilbene of grapes, may present in two isomeric forms, but only its *trans*-isomer has been identified in *Vitis vinifera* grapes (Langcake and Pryce, 1976; Jeandet *et al*., 1991; Vrhovsek *et al*., 1997). In wines, both *cis*- and *trans*-resveratrol were detected, with the latter predominant (Lamuela-Raventos *et al.*, 1995; Adrian *et al.*, 2000; Sun *et al.*, 2001c; Sun *et al.*, 2003b). The presence of *cis*-resveratrol in wines was generally considered to be due to the photochemical isomerization of partial *trans*- form during winemaking process (Jeandet *et al.*, 1995a; Roggero and Garcia-Parrilla, 1995). However, some authors suggested that *cis*-resveratrol might be present in grapes in combined form which could be liberated by hydrolysis during fermentation/maceration process of winemaking (Mattivi *et al.*, 1995; Vrhovsek *et al.*, 1997). More recently, *cis*-piceid was detected and quantified in skins (Sun *et al*., 2005c). Since skin tissue is the major source of such compounds to wine, the high level of stilbenes in grape skin has a practical importance in Enology. Grape seeds contain low amount of estibenes (Pezet and Cuenat, 1996), but considerable amount of these compounds may be found in grape stems (Bavaresco *et al*., 1997; Sun *et al*., 2003b), However, grape stems contribute little these compounds to stem-contact wine, due to their poor diffusion in must/wine (Sun *et al.*, 2001c, 2003b, 2005c). Furthermore, grape stems may be considered as sources of stilbenes for industrial utilization. More recently, Püssa *et al.* (2005) are successful in detection and quantification of dimer and trimer stilbenes in grape stems.

Extraction

Various solvents have been used for extraction of stilbenes from grape skins, among which ethyl acetate (Okuda and Yokotsuka, 1996; Landrault *et al*., 1999), acetone (Landrault *et al*., 1999), ethanol (Kanner *et al*., 2001), methanol (Landrault *et al*., 1999; Bais *et al*., 2000; Larronde *et al*., 2003) and methanol acidified by 0.1% HCl (Jeandet *et al*., 1995c) were the most used. From a

quantitative point of view, the results obtained using different extraction solvents stated could not be compared, one from another, because solvents of different natures would have different extractability. In order to establish a reliable quantitative method for extraction of stilbenes from grape skins, we have studied the extraction kinetics of different solvents and the maximum yields reached by each solvent were compared (Fig. 8).

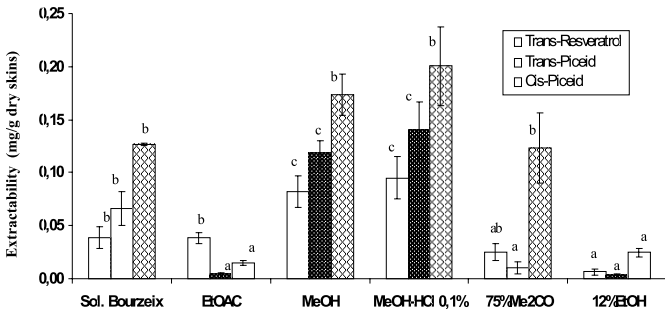


Figure. 8. - Extraction efficiencies of various solvents on grape skin stibenes. Vertical bars represent the standard deviation (n = 3). For the same compound, means followed by the same letter are not significantly different (LSD, 99%). Adapted from Sun *et al*. (2005c).

Eficiência de extracção de varios solvents para os estilbenos da película de uva. Barras verticais representam o desvio padrão (n = 3). Para o mesmo composto, as médias seguidas por a mesma letra indicam que não há diferença significativa (99%, LSD). Adaptado de Sun et al. (2005c).

Methanol acidified by 0.1% HCl (MeOH-0.1%HCl) and MeOH have higher extraction efficiencies than the combined extraction solvents proposed by Bourzeix *et al*. (1986) (Sol. Bourzeix) and 75% acetone in water (75%Me2CO). Both model solution of wine (12% EtOH) and Ethyl acetate (EtOAC) extracted only small amount of stilbenes.

Although the solvents of Bourzeix was verified to have much higher extraction efficiency on grape proanthocyanidins than any single solvent as described above, it has much lower extraction efficiency on stilbenes from grape skins than MeOH or MeOH-0.1%HCl. The reason for this may probably due to the high instability of stilbene molecules, which might be significantly degraded during the long and multi-step extraction process proposed by Bourzeix *et al* (1986), although much attention had been paid. In fact, when any single solvent was used for extraction of stilbebes in skins, the maximum yield was reached at less than 15 minutes. Thus, prolonged extraction time appeared not necessary. When MeOH or MeOH-0.1%HCl was used as extraction solvent, stilbene levels corresponding the maximum yield reached keep constant during the prolonged extraction time at least 48 hours, while for model wine solution, gradual decrease in stilbene levels may occur during this period. For example, the level of trans-resveratrol was slightly decreased during the period of

prolonged extraction using model solution of wine. The kinetic of extraction of stilbenes by model solution of wine would have a practical significance. The results obtained may suggest that once trans-resveratrol was diffused from grape skin to wine during fermentation, it would be gradually degraded throughout winemaking process. This suggestion has been further confirmed by monitoring the variation of this compound in red wine during conservation.

In a word, both MeOH and MeOH-HCl are good solvents for extraction of grape skin estilbenes. MeOH-HCl is preferred because it has the highest extraction efficiency. However, some precaution should be paid when MeOH-HCl is used because of the presence of acid in the extract, which should not be further concentrated to avoid hydrolysis of glucosylated forms of stilbenes.

Pre-purification and analysis

Both in grape skin extract and in wine, the concentration of stilbenes was generally much lower than those of other phenolic compounds such as catechins, proanthocyanidins and anthocyanins. Goldberg *et al.* (1996) analysed *cis*- and *trans*-resveratrol and their glucosides in nearly 700 commercial red wines from most of the world's areas of production. The concentrations of both resveratrol isomers in majority of red wines analysed were from 5 to 13 μmol/dm^3 (*i.e.* 1.14 to 2.96 mg/dm^3), and the concentrations of both glucosides, from 2 to 10 μmol/dm^3 (*i.e.* 0.78 to 3.90 mg/dm^3). Furthermore, the rosé wines and white wines contain much lower amounts of stilbenes than red wines (Romero-Pérez *et al.*, 1996a, 1996b). The *trans*-resveratrol concentrations in most white wines analysed by Siemann and Creasy (1992) were less than 0.03 mg/dm^3. Thus, direct injection of such samples to HPLC for quantitative analysis of these compounds is often not valid owing to important overlapping and/or to gradual baseline. As a consequence, pre° purification and concentration of the samples prior to HPLC analysis are necessary to ensure its accurate quantification.

In general, pre-purification and concentration of the samples for posterior analysis of stilbenes were carried out either by liquid-liquid extraction with ethyl acetate (Jeandet *et al*., 1991, 1995a,b; Siemann and Creasy, 1992) or by solid-phase extraction on C_{18} cartridge (Mattivi, 1993; Mattivi *et al*., 1995; Goldberg *et al*., 1995a,b, 1996; Soleas *et al*., 1995). However, validation of these methods was not provided. For this reason, we compared the following four methods for extraction of *trans*- and *cis*-resveratrol in red wine: liquid° liquid extraction with diethyl ether (Meth 1); liquid-liquid extraction with ethyl acetate (Meth 2); solid phase extraction on C_{18} cartridge using diethyl ether as eluant (Meth 3); solid phase extraction on C_{18} cartridge using ethyl acetate as eluant (Meth 4). The efficiencies of these sample preparation methods

were verified by HPLC analysis (Table IV) under the optimised chromatographic conditions as follows: column, 5-μm Lichrospher 100 RP 18; flow rate: 1,0 mL/min; injection volume, 30 μL; detection, 285 nm for *cis*-resveratrol and 307 nm for *trans*-resveratrol; column temperature, 30 ºC; gradient elution from 5% acetonitrile in water to 75% acetonitrile in water during 75 min was used, followed by washing and re-equilibrating column to initial condition.

Table IV

Efficiency of different pre-purification methods for wine resveratrol.

Eficiência de diferentes métodos de purificação prévia do resveratrol em vinhos.

	Meth 1		Meth 2		Meth 3		Meth 4	
	t-resv	*c*-resv	*t*-resv	*c*-resv	*t*-resv	*c*-resv	*t*-resv	*c*-resv
Repeatability (variation coefficient) (%)*	1.7	7.3	2.5	6.1	6.4	11.0	3.7	10.2
Extraction efficiency (%)**	102.0 ± 5.7	116.0 ± 10.5	106.8 ± 5.5	112.6 ± 9.1	63.7 ± 5.0	76.5 ± 9.3	64.6 ± 3.8	69.4 ± 8.0

Adapted from Sun et al. (2001c); *t*-resv *trans*-resveratrol; *c*-resv *cis*-resveratrol.
*Values obtained by 10 replications of the same wine.
**Mean value ± SD (n 5).

The methods of liquid-liquid extraction have the advantages over those of solid phase extraction as follows: linearity, precision and accuracy. On the other hand, the method of liquid-liquid extraction with diethyl ether has better selectivity than that with ethyl acetate. Furthermore, diethyl ether extracted, in addition to *cis*-and *trans*-resveratrol, only partially other monomeric phenols such as catechins and galic acid of wines, whereas ethyl acetate also extract many other phenolic compounds such as oligomeric procyanidins, and low amount of anthocyanins. In consequence, using diethyl ether liquid-liquid extraction method, the extracts of wine samples can be much concentrated prior to HPLC analysis, and thus permit quantifying the wines with trace amount of *cis*- and *trans*-resveratrol, particularly for rosé wine and white wine. However, our more recent work confirmed that diethyl ether does not extract glucosylated forms of resveratrol such as piceid and astringins but ethyl acetate does, with extraction efficiencies ranging from 98.2% to 108.3%. Thus, if not only *cis*- and *trans*- resveratrol, but also glucosylated stilbenes or other low-molecular phenolic compounds are interested, the liquid-liquid extraction with ethyl acetate should be used, instead of liquid-liquid extraction with diethyl ether. It should be also mentioned here that the gradient elution time we used is much longer than those previously published (Jeandet *et al*., 1995a,b). In fact, we have tested various elution programs of HPLC. In any case short elution time induced important overlapping between glucosylated forms of resveratrol (piceid and astringin) and other phenolic compounds,

thus piceid or astringin being probably overestimated, although the peak purities of *trans*- and *cis*-resveratrol were generally acceptable.

In conclusion, our recent work suggests the following procedures for quantitative analysis of grape skin stilbenes (Sun *et al*., 2005c): 100 grape berries are randomly harvested according to the standard method proposed by Bourzeix *et al*. (1986). After frozen, the skins of berry are manually and carefully separated, weighted, and lyophilized. The dry skins are weighted again before ground finely ($\phi \leq 1$ mm). 5g of skin powder is macerated in 100 ml of MeOH-0.1%HCl solution and the extraction is performed immediately with agitation, under nitrogen gas and in darkness. After 15 minutes of extraction, the extract is centrifuged at 1000g for 15 min and the supernatant is mixed with equal volume of water prior to evaporation to remove organic solvent (MeOH). The aqueous solution is extracted by three time of sequential liquid-liquid extraction with equal volume of ethyl acetate. If only resveratrol is interested, diethyl ether can be also used instead of ethyl acetate. The organic layer is evaporated to dryness, recovered by 2-5 ml of 50% ethanol in water, followed by HPLC analysis with elution program described above. The method, covering from skin extraction by MeOH-0.1%HCl, pre° purification using liquid-liquid extraction with ethyl acetate until HPLC analysis, has a very good repeatability, with coefficients of variation for *trans*° resveratrol, *trans*- and *cis*-piceid (3.25%, 3.38% and 1.59%, respectively) at the concentration > 10mg/kg much lower than the limit of that (15%) proposed by Monteiro and Bertrand (1994).

For analysis of wine stilbenes, 20 ml of red wine, 75 ml of rosé wine or 150 ml of white wine is directly extracted by three time of sequential liquid-liquid extraction with ethyl acetate. The organic layer is evaporated to dryness, recovered by 2 ml of 50% ethanol in water, followed by HPLC analysis under the optimized conditions.

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RESUMO

Revisão: extracção e análise quantitativa de proantocianidinas e estilbenos da uva e do vinho

Do ponto de vista de importância biológica, as proantocianidinas (taninos condensados) e os estilbenos (1,2-diarilletenos) são as duas classes de polifenóis mais importantes na uva e no vinho, o que tem chamado a muito atenção da comunidade científica internacional durante os últimos 15 anos, devido essencialmente aos potenciais efeitos benéficos destes compostos para a saúde humana, relativamente à sua acção protectiva contra a doença cardiovascular e a sua

capacidade de captar radicais livres de oxigénio. Numerosos trabalhos de investigação sobre as proantocianidinas e os estilbenos foram publicados, mas a maior parte deles têm incidido na identificação estrutural e nas actividades biológicas destes compostos. Porém, pouca atenção tem sido dispensada aos aspectos quantitativos e poucos métodos analíticos têm sido validados. Este artigo apresenta uma breve introdução sobre as proantocianidinas e os estilbenos da uva e do vinho e trabalhos realizados no nosso laboratório relativamente à extracção e análise quantitativa destes compostos.

RÉSUMÉ

Revue bibliographique: extraction et analyse quantitative des proanthocyanidines et des stilbenes du rasin et du vin

Du point de vue de l'importance biologique, les proanthocyanidines (tannins condensés) et les stilbènes (1,2-diarylethenes) sont les deux classes de polyphénols les plus importantes du rasin et du vin, qui ont fait beaucoup retenu l' attention de la communauté scientifique internationale pendant les dernières 15 années, principalement à cause des effets bénéfiques pour la santé humaine, relativement à leur action protective contre les maladies cardiovasculaires et à leur capacité de capture des radicaux libres ~~de~~ oxygènés. Bien que beaucoup de travaux scientifiques ont été publiés, le plupart de ces travaux ont été realisés sur l'identification structurale et les activités biologiques de ces composés. Peu attention a été prise sur l'aspect quantitatif et peu de méthodes analytiques ont été validées. Cet article présente en une brève introduction les connaissances actualisées sur les proanthocyanidines et les stilbenes du rasin et du vin, ainsi que les travaux réalisés dans notre laboratoire sur l'extraction et l'analyse quantitative de ces composés.

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