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# THE AROMA OF GRAPES

## I. EXTRACTION AND DETERMINATION OF FREE AND GLYCOSIDICALLY BOUND FRACTIONS OF SOME GRAPE AROMA COMPONENTS

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SUMMARY

A method of extraction and determination of free and glycosidically bound terpenols and of benzyl and 2-phenylethyl alcohols is suggested. The extraction was carried out by adsorbing these compounds on a non-ionic resin, Amberlite XAD-2, and then elution with various selective solvents. Free forms were directly determined by gas chromatography; glycosidically bound forms were first enzymatically hydrolysed to release aglycones. This method was applied to a number of mature grape varieties. The levels of glycosidically bound terpenols and aromatic alcohols were always high, usually much higher than the free forms. The varieties can be classified in two groups: those rich in free and bounds forms which give aromatic wines (Muscat varieties and Gewürztraminer), and those which contain small amounts of these compounds and which give more neutral wines.

## INTRODUCTION

It is known that the aromatic components of some grape varieties are present in the berry either in a free state or bound to sugars in the form of glycosides[1,2]. The free forms are present in different quantities in different varieties; in Muscat in particular, they form the major part of the aroma and consist essentially of terpenols in various states of oxidation and of terpenoid polyols[3–7]. It is generally simple to extract this free fraction and quantitate it[8–11]. The existence of a non-volatile, bound fraction was shown more recently and has now been characterized[1,7,12–15]; it is made up to disaccharide glycosides, namely α,L-arabinofuranosyl-β,D-glucopyranosides or α,L-rhamnofuranosyl-β,D-glucopyranosides, the aglycone of which can be a terpenol, a terpene diol, 2-phenylethanol or benzyl alcohol[11,13,14,16]. The specific extraction of these water-soluble compounds is more difficult as their concentration in grapes is low and they are admixed with a large quantity of other substances such as osidic and pectic compounds. In consequence, few quantitative data are available[11]. However, it is of great interest to determine this glycosidically bound fraction. For this

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reason, we have developed an extraction and analysis method to determine the free and bound terpenoid fractions in the same sample.

## EXPERIMENTAL

Various stationary phases have already been used to fractionate grape[1,11,16] and passion-fruit[17] glycosides (octadecyl bonded silica) for some flavanosides[18,19], some terpenoid derivatives[20] and for naringin and limonin from grapefruit juice[21] (Amberlite XAD-2). We chose the latter resin which possesses an excellent capacity for adsorption of free terpenols from grape juice[9]. Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) (50–80 mesh) was washed successively with methanol, acetonitrile and diethyl ether (each for 8 h); it was then dried and stored in methanol.

All the reagents used were analytical grade. The solvents were redistilled: ethyl acetate and methanol from potassium hydroxide, diethyl ether from iron sulphate, and pentane from potassium hydroxide and potassium permanganate. De-ionized water was used; it was distilled in the presence of potassium permanganate and then passed through an Amberlite XAD-2 column kept for this purpose.

Glycosidic substrates or glycosidasic extracts were prepared either from grapes or from various sources: glucosidase from Pectinol V.R. (Rohm), glucosidase from sweet almond (Koch-Light Labs.) and rhamnodiastase from buckwheat. Details have been given elsewhere[22].

### *Plant material*

The grape varieties studied here were harvested from the collections of the Agronomy Research Centre and of the Ecole Nationale Supérieure Agronomique in Montpellier. Fresh grapes, deep-frozen grapes and leaves or corresponding wines, were investigated.

*Juices and wines.* When fresh grapes were employed; these were cooled to 1°C immediately after picking; then crushed and pressed and the juice was centrifuged (9000 *g*, 15 min). All these operations were performed at 1°C. Sulphurdioxide (50 ppm) and sorbic acid (200 ppm) were added to the clarified juice. When analysis was to be carried out some time after picking, immediately after harvesting the grapes were deep-frozen at −20°C and stored at this temperature. They were thawed to 1°C just before analysis and then crushed in a whirlmixer; the pulp was filtered through gauze and centrifuged (9000 *g*, 15 min) at the same temperature. The clear juice was stabilized as previously described. Wines were diluted in an equal volume of water before analysis.

*Solids.* Leaves and grape solids (skins, pulp) were deep-frozen immediately and stored at −20°C until their extraction. This was carried out as follows: 50 g of plant material were immersed in liquid nitrogen and then crushed in a ball grinder, still under liquid nitrogen. Hot acetate buffer ($3 \cdot 10^{-2}$ *M*, pH 5.0, 150 ml) stored at 90°C was added. The operation was carried out in several stages to prevent a decrease in temperature and 0.1 *M* sodium hydroxide was added to maintain a pH of 5.0 (the volume of sodium hydroxide to be added was determined before using an aliquot of plant material). The mixture was homogenized, left to stand for 5 min at 90°C, cooled and then centrifuged (9000 *g*, 15 min, 2°C). The supernatant (about 120 ml) served for determination.

*Column preparation*

Amberlite XAD-2 resin suspended in methanol was poured into a glass column (35 × 1 cm I.D.) whose lower part was fitted with a PTFE tap and a glass-wool stopper. The packed column contained about 10 cm of resin. Several 25-ml volumes of methanol and then of diethyl ether were passed through it followed finally by 50 ml of water. The column was then ready for use.

*Fractionation and determination of free and bound fractions of the aroma*

A sample of 50–100 ml of juice, wine or plant extract was passed through the column with 10 μl of 0.1% solution of 2-octanol in ethanol, added as internal standard. The flow-rate was about 2–2.5 ml/min. The column was then rinsed with 50 ml of water to eliminate sugars, acids and other water-soluble compounds.

*Fractionation of the free fraction.* The free fraction of the aroma fixed on the column was eluted using 50 ml of pentane at a flow-rate of 2–2.5 ml/min. The pentane extract was dried with anhydrous sodium sulphate and then concentrated to a final volume of 50 μl by rectification at 40°C. The concentrate was used for gas chromatographic (GC) analysis.

*Fractionation of the bound fraction.* After the free fraction, the bound fraction was eluted using 50 ml of ethyl acetate. It was then dried with anhydrous sodium sulphate, filtered and concentrated, first to 1 ml under vacuum at 40°C, and then to dryness at 45°C using a stream of nitrogen.

*Hydrolysis of the bound fraction.* To the above dry glycoside extract was added 0.1 ml of $2 \cdot 10^{-1}$ *M*, citrate–phosphate buffer, pH 5.0. The mixture was washed four times using 0.1 ml of pentane to eliminate possible traces of the free fraction, using strong agitation in a whirlmixer. Pectinol solution (0.4 mg of Pectinol in 0.1 ml of $2 \cdot 10^{-1}$ *M*, citrate–phosphate buffer, pH 5.0) was then added to the extract. After agitation, the mixture was poured into a tube, hermetically sealed and placed in a water-bath for 12 h at 40°C. The medium was then extracted five times with 0.1 ml of pentane. After addition of 10 μl of 0.1% solution of 2-octanol in ethanol as internal standard, the solvent was eliminated by rectification at 40°C to give a final volume of 40 μl. The extract was then analysed by GC.

*Chromatographic determinations*

Terpenoid compounds and benzyl and 2-phenyl ethyl alcohols were determined using a Carlo Erba Fractovap gas chromatograph, Series 2900, equipped with a glass capillary column (69 m × 0.5 mm I.D.) coated with 0.3 μm of free fatty acid phase (FFAP) and connected to a Spectra-Physics SP 4000 Integrator. The fractionation was carried out first at a constant temperature of 70°C for 5 min, then with a temperature gradient from 70 to 180°C at a rate of 2°C/min and finally at a constant temperature of 180°C for 30 min. The carrier gas (hydrogen) flow-rate was 2.5 ml/min. The volumes of pentane extracts injected were between 2 and 3 μl. All data are in micrograms of volatile compounds per litre of juice or per kilogram of fresh plant material.

## RESULTS AND DISCUSSION

Amberlite XAD-2 resin displays extraction capacities similar to those of coated

TABLE I

HYDROLYSIS BY VARIOUS ENZYMATIC EXTRACTS WITH SIMILAR GLUCOSIDASIC ACTIVITY TOWARDS A GLYCOSIDIC EXTRACT FROM GRAPE

Results are given in μg of volatile compound released after enzymatic hydrolysis of a glycosidic extract from 1 l of Muscat juice.

| *Enzymatic system* | *Linalool* | *α-Terpineol* | *Citronellol* | *Nerol* | *Geraniol* | *Benzyl alcohol* | *2-Phenylethanol* | *Volatile compounds released (% of content released by Pectinol)* |
|---|---|---|---|---|---|---|---|---|
| β-Glucosidase from sweet almond (0.4 mg) | 29 | 13 | | 37 | 333 | 22 | 18 | 11.8 |
| Extract from Muscat grape (61 mg) | 9 | 10 | 14 | 135 | 419 | 19 | 57 | 17.3 |
| Extract from buckweat (2.3 mg) | 925 | 74 | 14 | 221 | 953 | 46 | 64 | 59.8 |
| Pectinol (0.4 mg) | 1368 | 64 | 29 | 601 | 1584 | 76 | 114 | 100 |

octadecyl silica. Furthermore, it has the advantage of being sold in large particle sizes; it is thus possible to use it in a wide preparative column at atmospheric pressure. The extraction yield of glycosides is much higher than that of solvent extraction techniques[2]; in addition, it is possible to eliminate numerous interfering substances such as sugars and acids by simple washing with water without any loss of terpenols and glycosides. These compounds are fixed entirely on the column: it was impossible, using GC to detect them in the column effluents and washing water. After elution, the recovery calculated from synthetized glycosides was between 90 and 100%[2]. With the conditions used, the volume of resin was sufficient to adsorb the free and bound fractions from at least 300 ml of juice; so there was no danger of saturating the adsorbent. With its rapidity and the possibility of use at low temperature, this technique limits the risks of artefacts which may be induced by the occurrence of natural enzymatic systems.

As regards solvents, the choice of pentane for free terpenol elution is the result of a compromise: a more polar solvent like dichloromethane gives a better elution of these compounds[9] but removes part of the glycosides. On the other hand, pentane does not elute glycosides but does not enable full recovery of free terpenols: depending on the compound, from 7 to 16% remains fixed on the column[9]. Ethyl acetate elutes glycosides as well as methanol but the former one is more selective.

The identity of the bound fraction was also investigated. The glycosidic part contains glucose, rhamnose and arabinose. The identity of the aglycones was determined after analysis of pentane extracts by gas chromatography–mass spectrometry (GC–MS)[2]; five terpenols were thus identified (linalool, α-terpineol, citronellol, nerol and geraniol) together with linalool pyranic oxide, one diol (3,7-dimethyl-1,5-octadiene-3,7-diol) and two aromatic alcohols (benzyl and 2-phenylethyl alcohols). These results confirm those of other authors[1,11,14].

As regards extraction from solid parts (leaves and grape solids), hot maceration was carried out at a high pH to avoid hydrolysis of glycosides[12,13]. A compromise between the duration and temperature of maceration was employed enable the solubilization of all undamaged free terpenols in the liquid medium.

It has been possible to determine the bound fraction of the aroma using glycoside hydrolysis followed by determination of the terpenoid aglycones released. Acid hydrolysis was discarded as it may induce considerable modification of the terpenol composition[12,13,17,23–26]. Enzymatic hydrolysis was chosen as the best method and the most effective enzymatic material was selected experimentally from among a number of extracts with glycoside activity (enzymatic grape extract, rhamnodiastase from buckwheat, sweet almond glucosidase, Pectinol); the activity of each preparation, all with a similar activity towards *p*-nitrophenyl-β,D-glucoside, was tested on the same glycosidic grape extract by measurement of the terpenols and aromatic alcohols released (Table I). Pectinol, which had already been used by others[11], was the most active, followed by buckwheat rhamnodiastase. Both preparations have particular affinity for glycosides, the aglycone of which is linalool; β-glucosidase and enzyme extract from grapes were less active, nevertheless a larger quantity of aglycone was released by the latter[22]. This phenomenon has already been noted[12] and is related to the nature of the glycosidic part of glycosides, comprising disaccharides which can be degraded by β-glucosidase[22]. The kinetic study of the enzymatic reaction with a grape glycosidic extract showed that hydrolysis was complete after 12 h of incubation

TABLE II

LEVELS OF BOUND AND FREE TERPENOLS AND AROMATIC ALCOHOLS IN VARIOUS GRAPE VARIETIES

Results are given as μg/l of juice.

| *Grape variety* | *Total terpenols* | | *Geraniol* | | *Linalool* | | *Nerol* | | *α-Terpineol* | | *Citronellol* | | *2-Phenylethanol* | | *Benzyl alcohol* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Bound* | *Free* | *Bound* | *Free* | *Bound* | *Free* | *Bound* | *Free* | *Bound* | *Free* | *Bound* | *Free* | *Bound* | *Free* | *Bound* | *Free* |
| Muscat of Alexandria | 4040 | 1513 | 1507 | 342 | 1839 | 1084 | 618 | 59 | 61 | 21 | 21 | 7.5 | 157 | 58 | 109 | 41 |
| Muscat of Frontignan | 1398 | 1640 | 396 | 107 | 207 | 1409 | 658 | 74 | 75 | 26 | 62 | 24 | 96 | 25 | 93 | 38 |
| Muscat of Hamburg | 1047 | 594 | 426 | 241 | 172 | 281 | 318 | 52 | 86 | – | 45 | 19.7 | 48 | 18 | 158 | 42 |
| Muscat Ottonel | 2873 | 1679 | 1291 | 172 | 722 | 1449 | 635 | 35 | 186 | 12 | 39 | 10.7 | 265 | 24 | 326 | 66 |
| Gewürztraminer | 4325 | 282 | 3356 | 218 | 22.5 | 5.6 | 617 | 43 | 183 | 3.2 | 146 | 12 | 159 | 37 | 185 | 35 |
| Riesling | 276 | 58 | 65 | 26 | 87 | 19.4 | 10.3 | 5.4 | 114 | 7.4 | – | – | 249 | 49 | 312 | 64 |
| Cabernet-Sauvignon | 26 | 5.3 | 12 | 3.6 | 4.2 | 1.7 | – | – | 9.9 | – | – | – | 88 | 16 | 144 | 38 |
| Carignane | 81 | 7.4 | 40 | 4.8 | 26 | 2.6 | 14.8 | – | – | – | – | – | 102 | 24 | 124 | 38 |
| Cinsaut | 314 | 13 | 69 | 13 | 5.4 | – | 6.9 | – | 233 | – | – | – | 177 | 9 | 135 | 8 |
| Clairette | 105 | 2.6 | 34 | 2.6 | 9.5 | – | 4 | – | 57 | – | – | – | 129 | 6 | 121 | 12 |
| Grenache | 71 | 11.8 | 40 | 5.2 | 5.4 | 6.6 | 8.2 | – | 17.4 | – | – | – | 81 | 18 | 160 | 52 |
| Picpoul | 105 | 5.8 | 25 | 5.8 | 2.2 | – | 3.0 | – | 75 | – | – | – | 163 | 38 | 181 | 46 |
| Syrah | 36 | 1.7 | 36 | 1.7 | – | – | – | – | – | – | – | – | 93 | 6 | 183 | 8 |
| Terret | 96 | – | 40 | – | 1.9 | – | 3.2 | – | 51 | – | – | – | 123 | 5 | 90 | 5 |
| Ugni blanc | 83 | 2.8 | 54 | 2.6 | 8.5 | 0.2 | 6.9 | – | 13.3 | – | – | – | 94 | 4 | 200 | 10 |

at pH 5.0 and at 40°C. The reproducibility of the method was tested on samples enriched with internal standard; the maximum deviation was 4–8% for the free fraction and 10–16% for the bound fraction.

*Application to the determination of free and bound fractions of aroma in mature grapes*

As shown in Table II, terpenols and aromatic alcohols have been found in all the mature grapes studied here. It is possible to classify them according to their free and bound terpenol contents. Some were rich in these compounds, particularly Muscat varieties which give aromatic wines; other were poor in terpenols and generally give wines with little aroma. Nevertheless, it was not possible to establish a classification according to the aromatic alcohol contents; the variations of the latter were not significant. Varieties with high bound terpenol contents generally also had high levels of free terpenols. Some had special characteristics such as Gewürztraminer which was rich in bound citronellol and poor in bound linalool; Cinsaut was similar with a high bound α-terpineol content. Among the bound terpenols, geraniol, linalool, nerol and α-terpineol are present in all the varieties; however, citronnellol is very often not present, either in free or bound form.

The results for free terpenols confirmed previous published data: aromatic grape varieties are characterized by high levels of linalool, nerol and geraniol and non-aromatic varieties by low levels of these compounds[8,27–29]. The latter did not contain α-terpineol or cintronellol whereas geraniol was present in all the varieties.

Variations of free and bound benzyl and 2-phenylethyl alcohols were low, as levels in these compounds are quite high in non-Muscat grape varieties in which terpenols are less abundant. These results give some information about the origin of these alcohols, until now considered as arising from fermentation, especially 2-phenylethanol[30,31]. The levels in grapes are however much smaller than those found in wines, 10–100 ppm[32].

The high proportion of bound terpenols compared to free terpenols, if generally confirmed by further studies, will be of considerable interest since it demonstrates the importance of the "hidden" aromatic potential in grapes. For example, in muscat of Alexandria and Riesling analysed here, the levels of bound aroma components were 3–5 times higher than those of free aroma components. In Muscat varieties, bound terpenols represented more than two thirds of the total terpenols. In Muscat of Frontignan this proportion was still about 50%, although it is the lowest because of the low linalool content in this variety. This "hidden" aroma represented 1–4 mg per litre of juice compared to 0.6–2 mg of free aroma. In varieties other than Muscats, these proportions were higher; in Gewürztraminer 94% of terpenols were present in the bound form. These results are fairly similar to those reported recently[33] in which the ratio of bound terpenols/free terpenols was greater than 1 in all varieties, including *Vitis vinifera* varieties.

With this evidence of a large glycosidically bound fraction of aroma, we are faced with the problem of the physiological significance of the glycosilation of terpenols in grape berries[34]. Certain technological applications might be possible, using different pathways[7,22,35] to break the bound forms and release free terpenols into the medium. This is all the more important since linalool and geraniol are among the most abundant bound terpenols, are two of the most aromatic ones and have a very low olfactive threshold[10]. Their contribution would probably be very positive

as regards aroma and quality of the results. However several conditions would need to be fulfilled: on the one hand, the bound terpenol levels must be sufficiently high (as in Muscat varieties and Gewürztraminer); on the other hand, it would be necessary for the bound aroma to be a faithful reflection of the free aroma (not the case for all the varieties studied especially Muscat of Frontignan). Finally the method of revelation of the aroma must not induce changes in it.

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