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# GAS-LIQUID CHROMATOGRAPHY AND WINE AROMA (¹)

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INVESTIGATIONS of wine aroma composition by classical chemical analytical techniques have not been very successful. The principal difficulty has been that the individual components responsible for the aroma are present in wines in such low concentrations that the older, standard analytical procedures have been unable to detect their presence. Attempts to isolate the aroma substances from the large amounts of water and ethanol of wines in the past have frequently caused destruction of the compounds sought or their alteration to a form not characteristic of the untreated wine. Distillation procedures have been particularly prone to cause both heat and acid mediated changes in the aroma components during the course of a lengthy fractionation. The result has been that wine analyses, in the classical sense, have measured the main constituents of wines — alcohol, sugars, acids — and

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usually by a procedure that depends upon a group-type reaction so that it was not possible to be certain of the presence of any one specific compound.

The development of chromatographic procedures in general, and of gas-liquid chromatography in particular, has greatly changed the earlier unsatisfactory state of our knowledge of wine aroma components. Gas-liquid chromatography has two great advantages that are particularly appropriate to aroma research — it can function as a very precise fractionating system thus permitting isolation of individual substances from very complicated mixtures and it is capable of detecting and measuring quantitatively very small amounts of organic compounds. Our knowledge of wine aromas thus effectively dates from about 1950 and most of the information has resulted from research done during the 60's.

Gas-liquid chromatography is only one type of chromatography. In this case the principle governing the technique is that different components of a mixture are likely to have different partition coefficients between a liquid phase and a gas phase in equilibrium with the particular liquid. Separation of components is accomplished by arranging that the liquid phase remain stationary while the gas phase is moved over successive increments of the liquid. Mechanically, the liquid phase is absorbed on an inert fine-meshed solid which is then packed into a relatively long, small-diameter tube. The solid is typically a diatomaceous earth filter-aid and the absorbed liquid a chemically inert, very high-boiling liquid such as the poly esters or silicone oils. The gas stream is caused to pass through the packed tube where it comes into intimate contact with the thin films of liquid on the grains of the inert solid packing material. As the partial pressure of any one sample component over the stationary liquid is a function of temperature as well as the nature of the sample and of the liquid phase, it is necessary to provide an oven for the column in which the temperature can be maintained at a constant value or in which a controlled program of temperature rise can be obtained.

While the packed column is the heart of the gas chromatograph, it is also necessary to provide a system for introducing the sample to be analyzed into the flowing gas stream, a system

to maintain a constant flow of carrier gas, and a system to detect and possibly collect the separated components of the sample.

Samples to be analyzed are normally liquids or solutions and are introduced by means of a hypodermic syringe through a silicone rubber septum into a vaporizing chamber through which the carrier gas flows on its way to the packed column. Ideally the sample is volatilized instantaneously and enters the packed column as the minimum volume of vapor corresponding to the volume of liquid injected. If the sample contains substances that are easily decomposable by heat, an alternative injection system may be employed in which the liquid sample is placed directly into the packing material of the head end of the column, thus avoiding exposure to the high temperature of the vaporization chamber.

The flow of the inert carrier gas through the packed column must be maintained at a constant optimum value for efficient operation of the gas chromatograph. Not only is the efficiency of the separations obtained a function of gas flow rate, but the sucessful behavior of the sample detection system is usually dependent upon a specific and constant gas flow. The resistance to gas flow of the packed column increases as the column temperature is raised with the result that for programmed temperature rise analyses it is necessary to provide special flow controllers in order to maintain a constant gas flow rate. Isothermal analyses, on the other hand, can be performed with only the simpler constant- pressure controllers to maintain a constant carrier gas flow rate.

To be a useful analytical tool the gas chromatograph must have some method of detecting the presence of separated components of the injected sample in the gas stream leaving the packed column. Although many different techniques for doing this have been devised, in practice one of two systems is generally used. The older system is based on measurement of the thermal conductivity of the gas stream leaving the analytical column. This technique has the great advantage that it does not destroy the samples, but it is somewhat less sensitive than the system based on measurement of the ions produced as the components in the gas stream are burned in a small hydrogen flame. The signal from the detector system is usually used to drive the pen of a recorder which then produces the typical gas chromatogram in the form of a succession of more or less sharp peaks as a function of time.

Every heat-stable, volatile organic compound has a specific time required to emerge from the gas chromatographic column. This value, called the retention time, is a function of the nature of the stationary absorbing liquid, as well as of the operating parameters of the analysis. Agreement of retention times between an unknown sample component and a known reference compound on analysis with two or more different stationary liquid phases of different polarity is evidence that the two substances are *likely* to be identical. Proof of identity depends upon collection of a portion of the unknown at the gas exit end of the packed column and determination that the infrared and mass spectra are identical with those of a portion of the reference compound similarly gas-chromatographed.

With the help of gas-chromatographic techniques the list of wine aroma compounds has been increased to nearly two hundred and is being continually augmented. Further, enologists are now able to understand much more about the biosynthesis of aroma components than was possible even a few years ago. The aroma and bouquet (components sensed by smell) in wines can arise from a number of sources — the grape, through action of yeast enzymes on sugars and other non-odorous solids, through enzymatic action on odorous volatiles of grapes, and through non-enzymatic chemical changes during cellaring and aging. Studies of aroma extracts from grapes and from wines of different types and ages have been most rewarding.

In addition to ethyl alcohol which, together with carbon dioxide is the principal product of the action of the yeast enzymes on the glucose and fructose of the grape, there are many more alcohols present in wine aroma. At least four of these, known as fusel oil, are formed during the course of the fermentation from the sugars or from certain of the amino acids in cases where there is an unbalanced mixture of amino acids naturally present. The pathway leads from sugar to pyruvate to 2-ketobutyrate, 2-ketoisovalerate, 2-ketoisocaproate, and 2-keto-3-methylvalerate. At this point the keto acid can either be aminated (from glutamic acid) to produce 2-aminobutyrate, valine, leucine, or isoleucine or it can be decarboxylated and reduced to yield n-propyl, isobutyl, isoamyl, or act. amyl alcohols. The fusel oil alcohols, which are present in wines at levels of from 200 to 400 mg per liter, are of importance in the aroma and flavor. Among the other alcohols present in wines, n-hexyl alcohol occurs in relatively high concentrations for a trace component. It apparently comes from the grapes rather than being produced by enzymes from sugars although it may also result from breakdown of unsaturated acids during vinification. There are many more alcohols present in only very minute amounts. A number of these remain only partially identified, and their possible contribution to the aroma of the wine unknown.

If strong reducing conditions are not established during the fermentation or if the wine is aerated excessively during its cellaring period, higher than the normal trace concentrations of aldehydes corresponding to many of the alcohols present will accumulate. Further, during the period of aging of the wine in oak containers a small amount of aromatic aldehydes will be extracted from the wood. As most of these aldehydes have low sensory thresholds, even the small amounts present in wines are very likely of importance in the aroma.

The volatile organic acids have low sensory thresholds and there are small amounts of a large number of them present in wines. Although small amounts of the saturated, straight-chain, monocarboxylic acids are naturally present in the grapes, a much greater amount derives from the yeasts. Most wines seem to contain the $C_6$, $C_8$, and $C_{10}$ acids in the highest concentrations. These can logically be expected to result from leakage from the enzyme surface during the normal process of building palmitic and stearic acids through condensations of acyl-coenzyme A units with malonyl-coenzyme A. Certain of the unsaturated and hydroxy acids may also arise through this mechanism. Branched chain and aromatic acids probably arise from oxidations of the alcohols and aldehydes of corresponding carbon skeletons.

In case the acyl-coenzyme A unit reacts with an alcohol instead of water upon its escape from the enzyme surface, an ester rather than and acid is the product. Grapes are found in general to contain relatively few and low concentrations of esters, while wines immediately after fermentation are found to be rich in a wide variety of esters. Most of the esters are ethyl esters, which is in accord with the suggested mode of their formation. The finding that the ethyl acid malate of film sherry is 100 % the single isomer with the hydroxyl group on the carbon atom adjacent to the free acid group suggests that the esters of the dibasic acids are also enzymatically formed, although the analyses of very old wines by Ribéreau-Gayon and Peynaud indicates a slow hydrogen ion catalyzed chemical esterification is active too. The ester usually present in highest concentration, ethyl acetate, contribues general fruitiness to wines when present in relatively small amounts but is the characteristic odor of bacterially acetified wines and a mark of spoilage. The importance of the other esters to the aroma of wines is still an open question, and one meriting further study.

If the acetyl-coenzyme A complex should react with an amine rather than water or an alcohol one obtains the N-substituted acetamides that are found in certain sherries. Although N-ethyl-acetamide was found in White Riesling grapes, N-isoamyl- and N-(2-phenethyl)-acetamides were found to be components of film sherry made from Palomino grapes. These compounds do not have low sensory thresholds and are therefore probably not of significance to the wine aroma. Ethyl-, isoamyl-, and 2-phenethylamine have been reported present in low concentrations in wines, thus making the suggested route of formation of N-substituted acetamides theoretically possible.

Analysis of a sample of submerged-culture flor sherry has shown the presence of a number of complex acetals. In addition to the diethyl acetal of acetaldehyde usually found in wines, all simple and mixed combinations of ethanol, 2-methybutanol, 3-methybutanol, and 2-phenethanol with acetaldehyde were isolated and identified. As these compounds have very intense and distinctive odors it is believed that they are of significance in the make-up of flor sherry aroma. Another acetal of interest from the chemical point of view is 2,4,5-trimethyl-1,3-dioxolane, the product of reacting an acetaldehyde molecule with a molecule of butanediol. Significant amounts of this acetal are present in flor sherries. Acetal production is a hydrogen ion catalyzed reaction and thus should proceed at a low rate at the pH of wines. It is also possible, of course, that the yeast cell contains enzymes mediating this reaction.

Lactones are internal esters in which the carboxylic acid group has reacted with a hydroxy group, usually on the fourth or fifth carbon atom, to form a five or six membered ring. Wines have been known for some time to contain the simplest one of the five membered ring series, gamma-butyrolactone. In sherries this lactone is apparently in equilibrium with small amounts of the free acid, 4-hydroxybutyric acid, and with the ethyl ester of the acid. Sherries contain a number of other gamma lactones among which pantoyl lactone and 4,5-dihydroxyhexanoic acid lactone are of considerable interest, the former because of its close relationship to the vitamin, pantothenic acid, and the latter because of its physiological activity. Gamma-butyrolactone, itself, is some ten times as effective a physiological depressant as is ethanol. Gamma-butyrolactone has a high sensory threshold, but certain of the 4-substituted gamma lactones have very low thresholds and are important components of flavoring mixtures. So far, none of the six-membered ring delta lactones has been identified in wines, but there seems no reason why they should not be present.

A few examples of aroma components of functionality other than those already discussed have been encountered in studies with wines. These include a few ethers, some hydrocarbons, some sulfur containing compounds, and probably some nitrogen containing substances in addition to the amides and amines. There are, no doubt, many more of these compounds and others awaiting isolation and identification.

It is interesting that no one of the fine wines of the world has been found to contain a single compound that is responsible for the characteristic aroma and bouquet. Rather, wine aroma and bouquet seem to be the result of the presence of a very large number of different chemical entities, each present in a rather definite concentration range. Investigations to date have sketched in the general outline of aroma and bouquet composition. Much critical detail remains to be filled

in. The procedures of gas-chromatography, coupled with infrared, nuclear magnetic resonance, and mass spectrometry, and the intelligent application of microchemical techniques should complete the picture in the not too distant future.

## SUMÁRIO

Neste trabalho aponta-se o quase-fracasso dos métodos de análise clássicos aplicados ao estudo dos aromas dos vinhos, devido à baixa concentração dos compostos químicos que os causam e também às decomposições ou alterações mais ou menos profundas que nestes provocam, em especial os métodos por destilação.

Refere-se, por outro lado, a vasta superioridade dos métodos de cromatografia em fase gasosa, desenvolvidos e aplicados de forma cada vez mais ampla nos últimos vinte anos.

Descrevem-se, de forma sucinta, as bases e modo de aplicação destes métodos susceptíveis de levar à obtenção dos melhores resultados.

Graças às técnicas de cromatografia em fase gasosa, o número de componentes conhecidos do aroma do vinho subiu, nos últimos anos, de poucos até cerca de duzentos, número este que está em constante aumento, o que pôs os enólogos em situação de compreenderem muito mais a fundo a biosíntese dos compostos em causa. Têm sido em especial remuneradores os estudos que incidiram sobre mostos e vinhos de diversos tipos e idades.

Acerca dos compostos importantes para o aroma e perfume do vinho que pela sua concentração muito baixa, muitas vezes unida a grande instabilidade, só puderam ser estudados com segurança pelos recentes métodos da cromatografia em fase gasosa, apresenta-se a seguinte lista:

- álcoois, diferentes do álcool etílico, formados durante a fermentação;
- aldeídos, aromáticos ou não;
- ácidos orgânicos voláteis;
- esteres, etílicos na sua maioria, cujo estudo merece bem ser aprofundado;
- acetamidas;

- acetais;
- lactonas $\gamma$;
- esteres, hidrocarbonetos, compostos sulfurados e compostos azotados diferentes das aminas e das amidas,

descrevendo-se também resumidamente a natureza e modo de formação de alguns deles.

Muitos mais compostos desta e de outra natureza aguardam isolamento e identificação.

Em conclusão, faz-se ver que nenhum vinho de qualidade tem o seu aroma e perfume característico dependente dum único composto, parecendo antes resultar da presença, em concentrações definidas, de grande número de compostos diferentes. As investigações até à data concluídas permitiram conhecer, com segurança, os traços fundamentais da composição geral do aroma e perfume, mas ficam ainda importantes pormenores por completar. Os processos de cromatografia em fase gasosa associados às espectrometrias de infravermelhos, de ressonância nuclear magnética e de massa, bem como a inteligente aplicação das técnicas microquímicas deve completar os conhecimentos mais importantes neste campo, num futuro não muito distante.

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