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GAS–LIQUID CHROMATOGRAPHIC AND MASS SPECTROMETRIC IDENTIFICATION OF ANTHOCYANIDINS

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SUMMARY

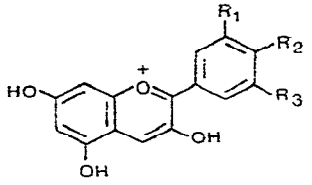
A method is described for the identification of anthocyanidins by gas–liquid chromatography and mass spectrometry. Anthocyanidins, when treated with trimethylchlorosilane and hexamethyldisilazane, yield volatile compounds whose nature is discussed on the basis of their high-resolution mass spectra, obtained by a direct inlet system, and nuclear magnetic resonance spectra. A facile thermal elimination of trimethylsilanol occurs if these products are analyzed by gas–liquid chromatography–mass spectrometry, with the consequent formation of quinoline derivatives. The latter give rise to well-separated gas chromatographic peaks and show fragmentation patterns which can be employed to define the structure of anthocyanidins.

INTRODUCTION

The anthocyanins are the most important and widespread group of colouring matters in plants and are known to be responsable for the colours of the flowers and fruits of plants. Chemically, they contain a polyhydroxyflavylium cation, whose colour depends on the location of the hydroxyl groups and the eventual presence of glycosidic residues. The nature of these compounds is usually defined by observing their R_F value by paper chromatography (PC) or by thin-layer chromatography (TLC)[1], and by considering their UV absorption maxima[1,2]. Characterization of an anthocyanin can also be achieved, after removal of the sugar moieties, by identification of its aglycone (anthocyanidin). In spite of the efforts of a number of workers[3,4], no successful results have been obtained by using gas chromatography (GC) for the identification of the anthocyanidins.

The necessity of characterizing these substances more precisely has encouraged us to seek new methods of investigation, and here we refer to the possibility of identifying single anthocyanidins and their mixtures by mass spectrometry (MS) and gas–liquid chromatography (GLC)–mass spectrometry. The anthocyanidins themselves cannot be analyzed by means of MS. This technique is hampered by the

limited volatiiity of these compounds. In this paper it will be shown that anthocyanidins can be converted into volatile derivatives by reaction with trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) and, as a consequence, can be analyzed by GLC and GLC–MS.



	R_1	R_2	R_3
cyanidin	OH	OH	H
delphinidin	OH	OH	OH
malvidin	OCH_3	OH	OCH_3
petunidin	OH	OH	OCH_3

EXPERIMENTAL

Materials

Four of the most comman anthocyanidins, *i.e.*, cyanidin, delphinidin, malvidin and petunidin, were used as obtained in our laboratories. TMCS and HMDS were obtained from Pierce (Rockford, Ill.. U.S.A.).

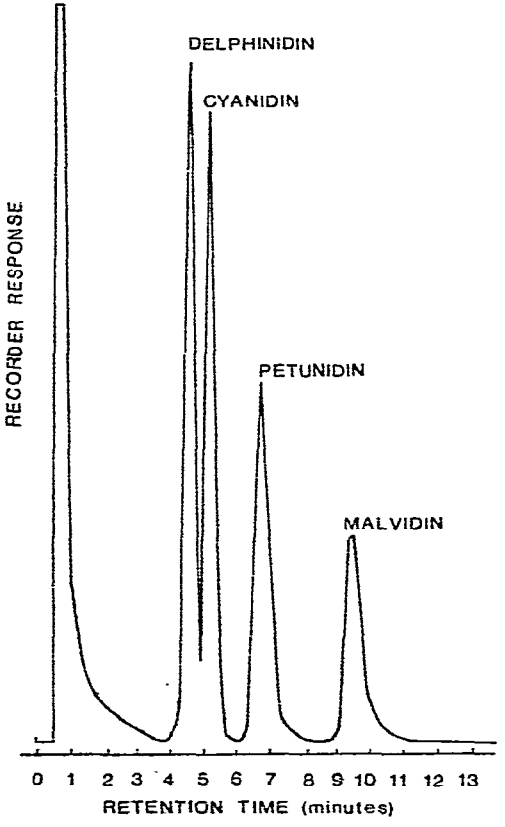


Fig. 1. Gas chromatogram of anthocyanidins after reaction with TMCS and HMDS.

Gas chromatographic analysis

The analysis was carried out on 0.1 μl of the solution obtained on heating a mixture of fine anthocyanidin powders (total amount, 5 mg) at 80° with 0.1 ml of TMCS and 0.2 ml of HMDS in a screw-capped vial with PTFE cap liners until complete dissolution and discolouring were obtained. A Varian 1400 aerograph gas chromatograph, equipped with a hydrogen flame ionization detector (FID), was used. The column (2 m × 2 mm) of coiled silanized tubes contained 3% OV-225 liquid phase loaded on silanized Chromosorb WHP (100–120 mesh). The oven temperature was 270°. The flow-rate of the carrier gas (helium) was 30 ml/min.

Fig. 1 shows a GLC separation of delphinidin, cyanidin, malvidin and petunidin. The four peaks were identified by comparison with the retention times of the pure individual products.

The silylation reaction may also be carried out by dissolving the sample in dimethyl sulphoxide (DMSO)–dioxane (1:1) (silylation grade, Pierce).

Mass spectrometry

GLC–MS. The mass spectra of the GLC peaks corresponding to the four anthocyanidins were recorded on a Varian MAT Model CH7 mass spectrometer combined with the Varian gas chromatograph. All of the spectra were measured at 70 eV, the temperature of the two-stage jet separator was 270° and the temperature of the source was *ca.* 200°.

Direct inlet system MS. The mass spectra of the pure individual anthocyanidins obtained by the direct-inlet system (DIS) were recorded on a high-resolution mass spectrometer (Varian MAT Model 711). For this purpose, a small quantity of delphinidin chloride (2 mg) was treated as previously with the silylating agents. The excess of reagents was evaporated under vacuum at room temperature until a thick liquid, almost colourless and suitable for analysis by DIS, was formed. It is important that a small quantity of the silylating reagents is preserved with the sample, and that the analysis is carried out as soon as possible, in order to avoid the formation of a monodesilylated product. The source temperature was 90° and the vaporization temperature was 70°.

Nuclear magnetic resonance (NMR) spectra

The NMR spectra were recorded on a Perkin-Elmer R24 60-MHz instrument. 70 mg of freshly crystallized delphinidin chloride were silylated with 1.5 ml of TMCS and 2 ml of HMDS. After heating at 80° in a screw-capped reaction vial for 20 min, ammonium chloride was filtered off and the clear solution was concentrated. A suitable quantity of [^{2}H]chloroform was then added.

RESULTS AND DISCUSSION

The mass spectra corresponding to the four peaks obtained by silylation and GLC separation of the anthocyanidin mixture are shown in Fig. 2. The spectra are characterized by a very intense molecular peak accompanied by much less intense peaks due to fragmentation ions. It is to be noted that in each case the molecular ion has an odd value of *m/e*. The presence of nitrogen in the molecules was therefore considered. DIS high-resolution measurements of the spectrum of silylated delphinidin

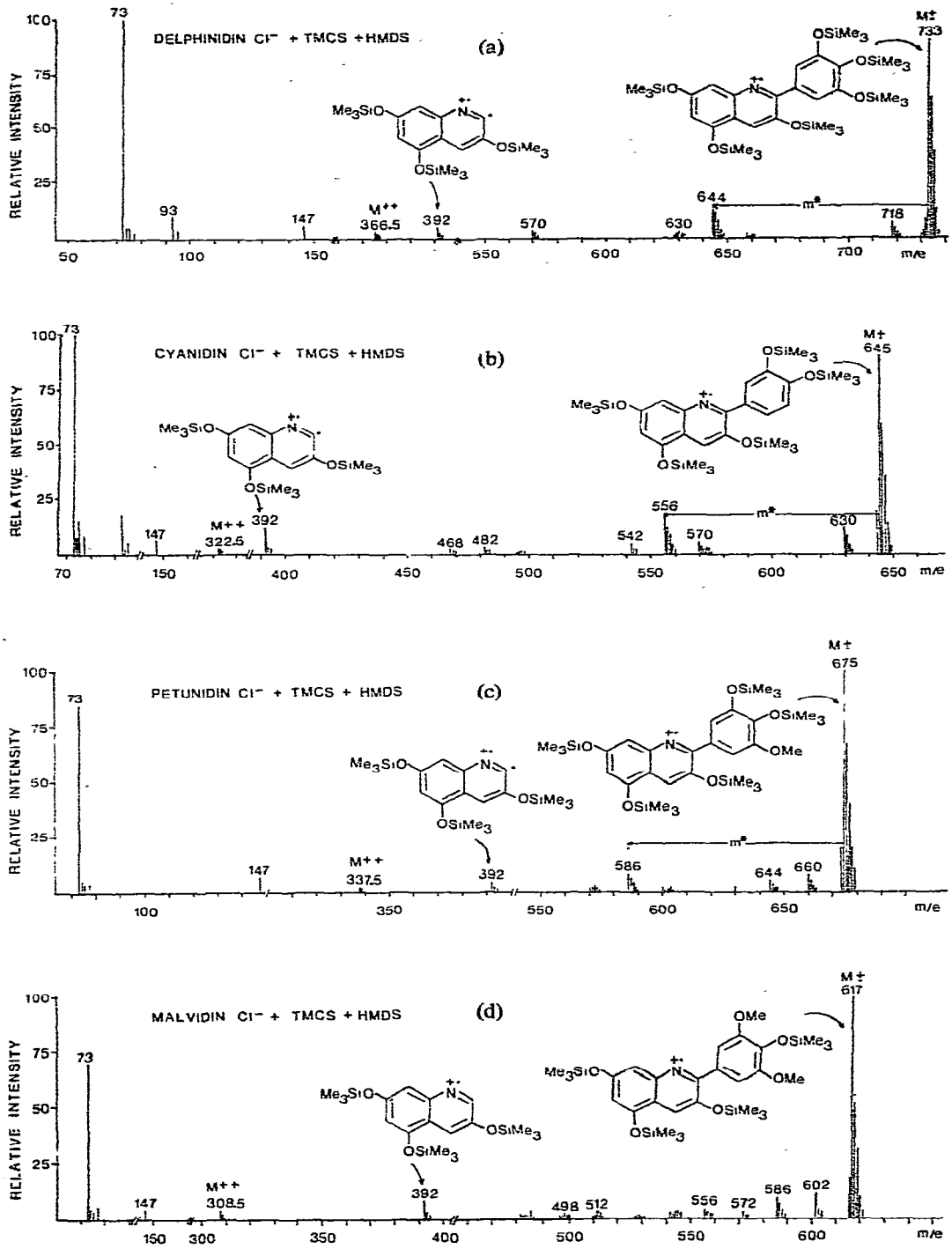


Fig. 2. Mass spectra of the GLC peaks of the anthocyanidins after treatment with TMCS and HMDS.

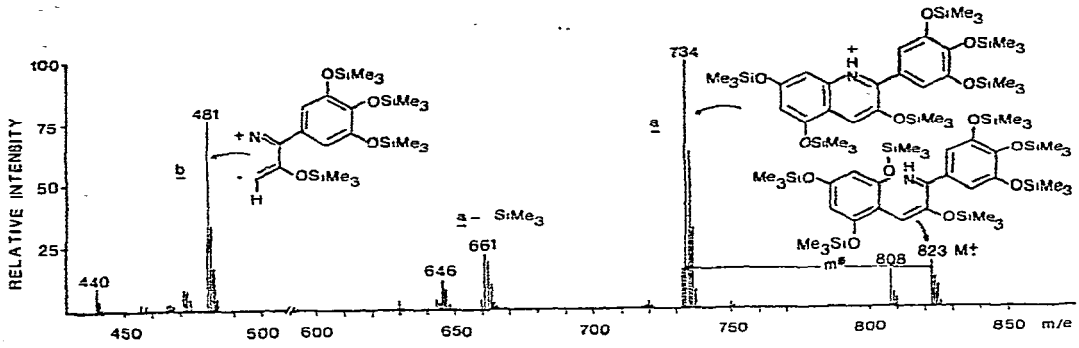
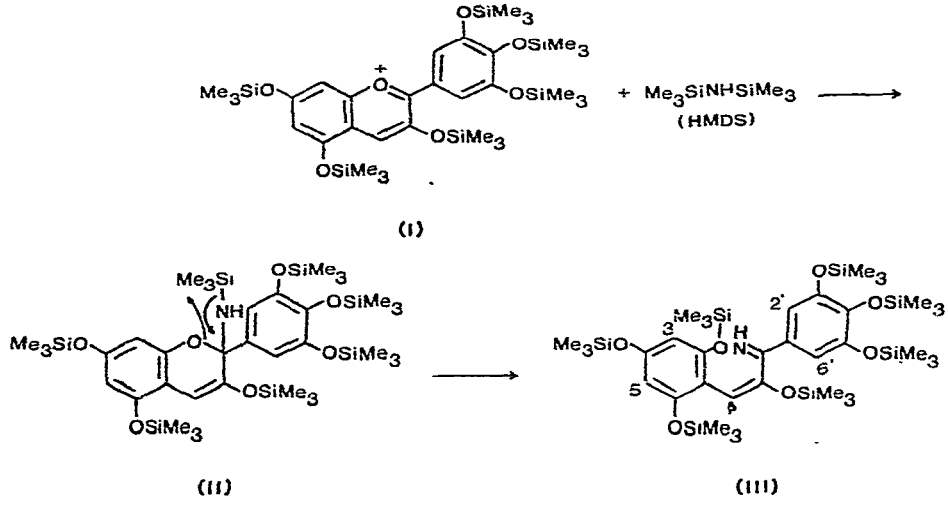


Fig. 3. High-resolution mass spectrum of silylated delphinidin chloride obtained by DIS. Resolution, 20,000.

chloride (Fig. 3) also show that the silylation reaction results in the introduction of a nitrogen atom. However, this type of analysis furnishes a completely different spectrum to that obtained after GC analysis (Fig. 2). The molecular ion is much less intense and occurs at *m/e* 823. This ion possesses the empirical formula $C_{36}H_{69}NO_7Si_7$ which corresponds to the silylation of all of the hydroxyl groups of the delphinidin and to the introduction of a Me_3SiNH group.



Scheme I

Taking into account the chemical properties of oxonium ions[5], structure II could be assigned to this derivative; however, in our opinion, the alternative structure III, which can arise from II through the rearrangement reaction shown in Scheme I, is in greater accord with the spectroscopic properties. The ^{1}H-NMR spectrum (Fig. 4) of the derivative shows that the two C-3 and C-5 protons are equivalent and give a singlet resonance at 5.65 ppm. The low-field resonance of the NH group is also in agreement with the structure III. The spectrum shows splitting of the signals, due

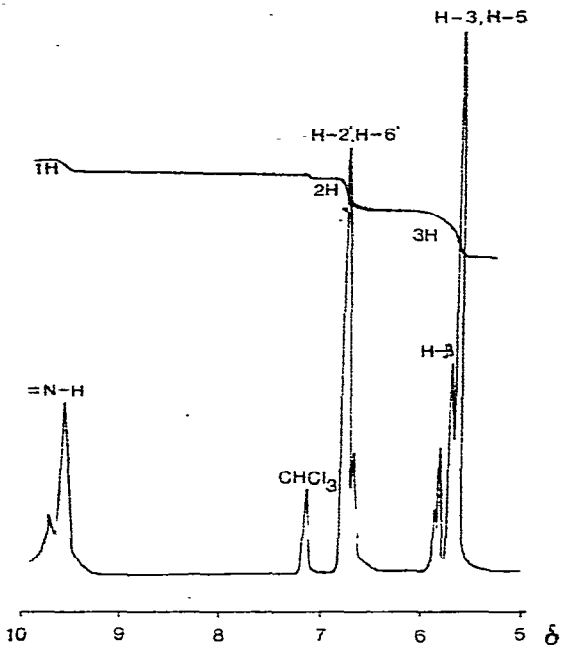


Fig. 4. 60-MHz NMR spectrum of delphinidin chloride after treatment with TMCS and HMDS.

probably to the steric hindrance of the bulky substituents to the interconversion of the rotational isomers. In addition, the high-resolution mass spectrum is well explained on the basis of a structure of this type. Apart from the ions at *m/e* 823 (M^+) and 808 ($M^+ - CH_3$), the mass spectrum shows an intense (100%) peak at *m/e* 734 which corresponds to the very stable ion (*a*) arising from the loss of the O-trimethylsilyl group from C-2. The fragment at *m/e* 481 is due to a radical ion (*b*). The same fragmentation pattern was exhibited by the other anthocyanidins (Table I).

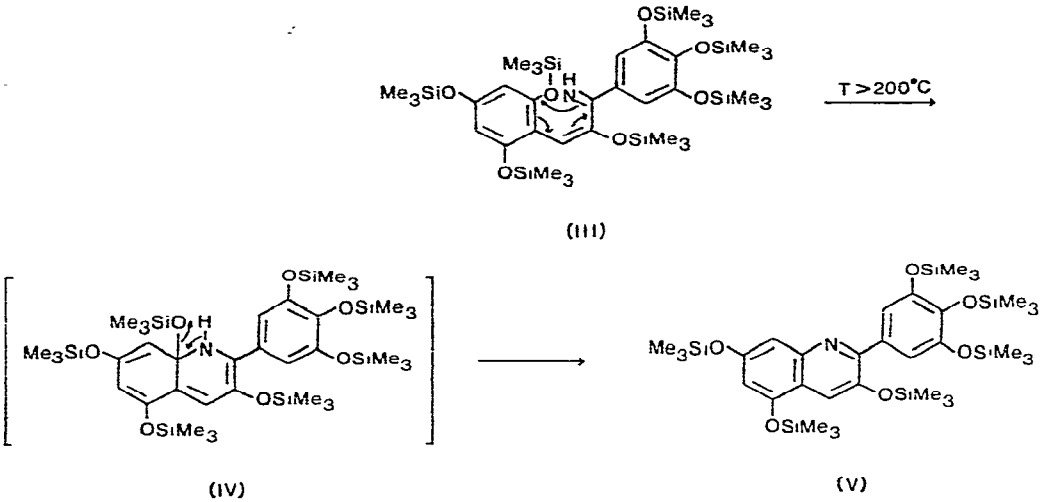
It still remains to explain the difference between the spectra obtained by DIS and after GLC analysis. It should be noted that the samples are subjected to different thermal conditions by the two techniques. The sample, analyzed by DIS, evaporates

TABLE I

FRAGMENTATION PATTERNS OF SOME SILYLATED ANTHOCYANIDINS

Silylated compound	M^+		$(M^+ - Me)$		*a*		*(a−OMe)*		*(a−SiMe₃)*		*b*	
	m/e	*I(%)*	*m/e*	*I(%)*	*m/e*	*I(%)*	*m/e*	*I(%)*	*m/e*	*I(%)*	*m/e*	*I(%)*
Cyanidin chloride	735	27	720	15	646	100	—	—	573	17	393	63
Malvidin chloride	707	43	692	17	618	100	587	5	545	6	365	70
Petunidin chloride	765	29	750	17	676	100	645	4	603	7	423	95
Delphinidin chloride	823	25	808	18	734	100	—	—	661	21	481	81

at 70°, whereas when analyzed by GLC it is subjected to a temperature higher than 200°. When the solution obtained on treatment of a anthocyanidin with the silylating agents was progressively heated under vacuum to 220° and then analyzed by DIS, a mass spectrum identical to that obtained by GLC–MS was found. The employment of the high-resolution MS indicates that, in the case of delphinidin, $C_{33}H_{59}NO_6Si_6$ is the elemental composition of the molecular ion, and that the high temperature causes the elimination of a molecule of trimethylsilanol as indicated in Scheme II.



Scheme II

The quinoline-like nature of the derivative V is supported by its mass spectrum. The ion at *m/e* 392, which occurs in all the anthocyanidins examined, has the formula $C_{18}H_{30}NO_3Si_3$ and possesses the structure indicated in the spectrum. The other minor peaks were also investigated by means of the high-resolution instrument. The peaks at *m/e* [M−75] arise through the loss of a C_2H_7SiO radical, the ion at *m/e* [M−89] from the loss of a Me_3SiO radical, the ion at *m/e* [M−103] from the loss of one Me_3Si and two methyl groups and the ion at *m/e* [M−163] from the loss of C_2H_7SiO, Me_3Si and methyl radicals.

The employment of other silylating agents such as N,O-bis(trimethylsilyl)-acetamide and N-trimethylsilylimidazole gives rise to more than one chromatographic peak and therefore cannot be used for GLC analysis of anthocyanidins.

CONCLUSION

The reaction of HMDS with the anthocyanidins gives rise to an open form (III) which may be characterized by NMR and MS analysis at low temperature. If this product is analyzed by GLC, the high temperature of the column causes the formation of a quinoline derivative (V), which is of equal use in the characterization of the initial anthocyanidin. All of these reactions, if carried out as mentioned in the Experimental section, produce one single product which can be easily identified by GLC–MS analysis.

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