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IDENTIFICATION OF ANTHOCYANINS BY GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

A method is described for the separation and identification of anthocyanins by gas–liquid chromatography and mass spectrometry. Treatment of anthocyanins with trimethylchlorosilane and hexamethyldisilazane yields nitrogen-containing derivatives which, after the injection into the gas chromatograph, produce quinoline-like compounds. The latter give rise to well separated gas chromatographic peaks and show fragmentation patterns that are useful for the identification of the starting material.

INTRODUCTION

Anthocyanins are usually identified by observing their R_F values in paper or thin-layer chromatography and by considering their UV absorption maxima. Recently, we have proposed the use of gas–liquid chromatography (GLC) as an additional, very simple tool both for the identification and for checking the purity of their aglycones, the anthocyanidins[1]. Most important, we have also found that this technique can be used satisfactorily for quantitative evaluations.

If a mass spectrometer is coupled to the gas chromatograph, electron-impact mass spectrometry serves as a valuable aid in determining the structures of the anthocyanidins, as information about oxygenation patterns and the location of hydroxyl, methoxyl and/or acyloxyl groups on the A and B rings of the anthocyanidin skeleton can be obtained.

The same technique has now been applied successfully to the anthocyanins, and the results are reported in this paper.

EXPERIMENTAL

Materials

Some of the most common anthocyanins were used as received in our laboratories. Trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS), tetrahydrofuran (THF) and dimethyl sulphoxide (DMSO) were obtained from Pierce (Rockford, Ill., U.S.A.).

Gas–liquid chromatographic analysis

The analysis was carried out on 1 μl of the solution obtained on heating finely powdered anthocyanins (1 mg) at 80° with a mixture of 0.1 ml of TMCS, 0.2 ml of HMDS, 100 μl of DMSO and 150 μl of THF in a screw-capped vial with PTFE cap liners until complete dissolution and decoloration were obtained. A Varian Aerograph 1400 gas chromatograph, equipped with a hydrogen flame-ionization detector (FID), was used. The column (0.5 m × 2 mm) of coiled silanized tubes contained 0.5% OV-101 liquid phase loaded on silanized Chromosorb W HP (100–120 mesh). The temperature was programmed from 200° to 320° at the rate of 10°/min. The flow-rate of the carrier gas (helium) was 30 ml/min.

Gas–liquid chromatography–mass spectrometry

The mass spectra of the GLC peaks corresponding to the anthocyanins were recorded on a Varian-MAT Model CH7 mass spectrometer combined with the Varian gas chromatograph. All of the spectra were measured at an ionization potential of 70 eV and a trap current of 60 μA. The two-stage jet separator was maintained at 350° and the temperature of the source was *ca.* 250°.

RESULTS AND DISCUSSION

Treatment of an anthocyanidin with TMCS and HMDS provides a volatile nitrogen-containing derivative, which arises from nucleophilic attack of an $NHSiMe_3$ grouping at position 2 and rearrangement of the $SiMe_3$ group from the nitrogen atom to the pyranyl oxygen atom. As an example, the process for the anthocyanidin petunidin (1) is reported in Fig. 1.

If the derivative 2 is analysed by GLC, an elimination reaction takes place

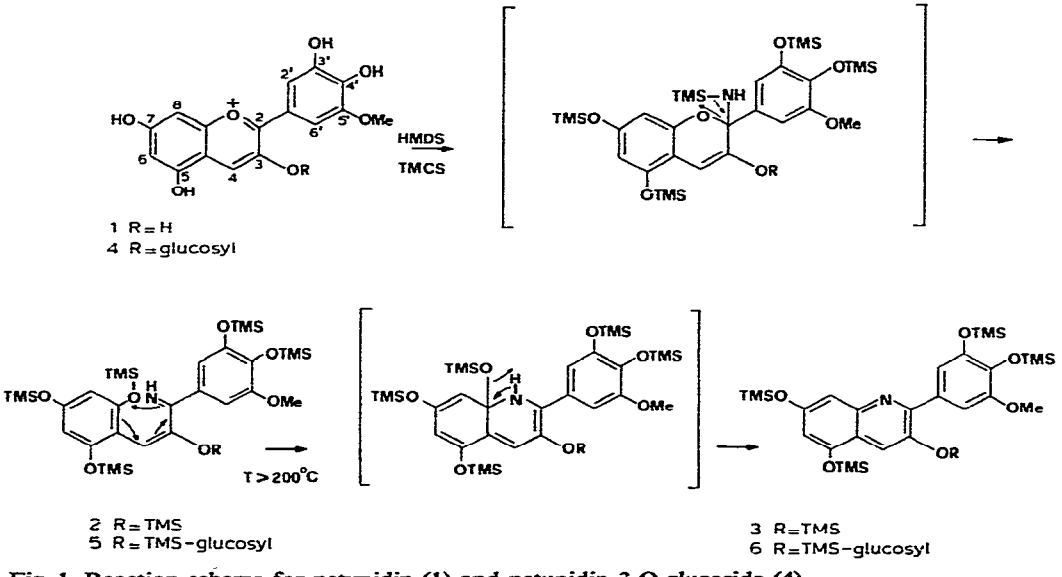


Fig. 1. Reaction scheme for petunidin (1) and petunidin 3-O-glucoside (4).

and, as soon as the sample is injected into the injection port, a compound with a quinoline-like structure (3) and displaying a sharp peak is obtained[1].

The behaviour of anthocyanins is essentially the same. For instance, petunidin 3-O-glucoside (4) gives rise, if the analysis is carried out according to the procedure described under Experimental, to only one peak with a retention time of 7.5 min.

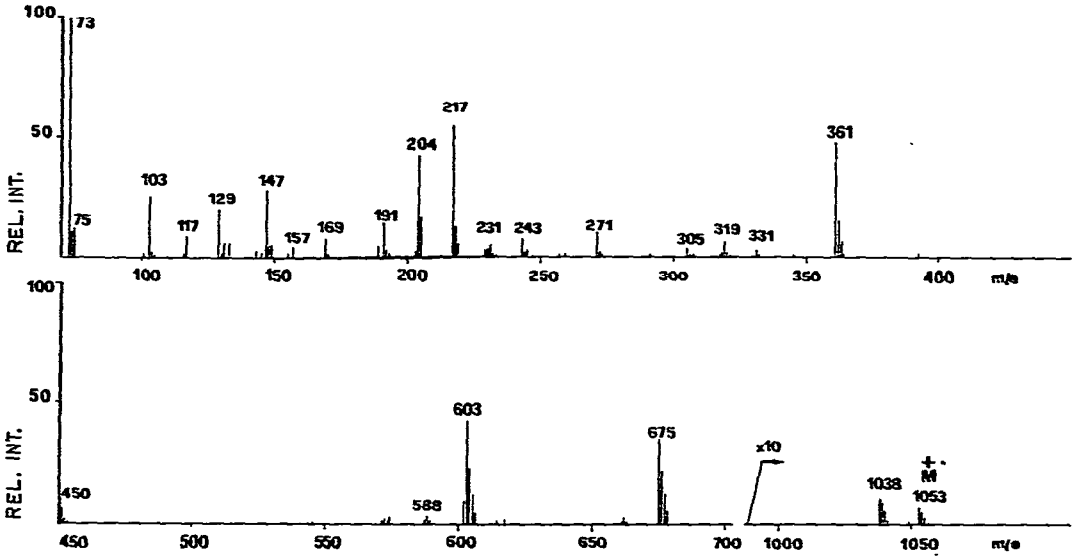
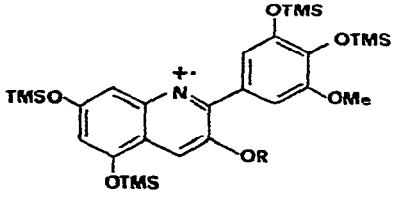


Fig. 2. Mass spectrum of derivative 6 (petunidin 3-O-glucoside + HMDS + TMCS).

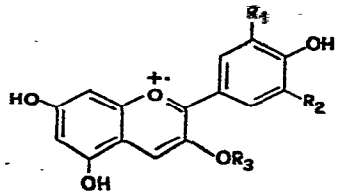
The mass spectrum of the peak is shown in Fig. 2 and agrees with the expected structure 6. In fact, apart from the fragment ions due to the decomposition of the glucopyranosyl grouping at *m/e* 450, 361, 332, 331, 319, 305, 289, 271, 217, 204, 191 and 103, the origin of which is well known[2], it contains a molecular ion peak at *m/e* 1053, as required by structure 6. Two intense peaks at *m/e* 603 and 675 are also discernible. These two ions are derived from the loss of the TMS-glucosyl residue followed by migration of H and TMS radicals, respectively, and therefore must be attributed the structures (a) and (b):



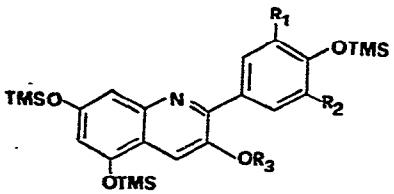
(a) R = H

(b) R = TMS

All of the monoglucosyl derivatives of anthocyanidins exhibit similar behaviour. For instance, the 3-O-glucosides at cyanidin (7) and malvidin (8) give rise to the quinoline derivatives 9 and 10, which have retention times of 6.8 and 7.9 min, respectively. Their MS spectra are reported in Figs. 3 and 4 and can be easily interpreted through comparison with the spectrum of the derivative 6.



(7) $R_1 = OH$ $R_2 = H$ $R_3 =$ glucosyl
(8) $R_1 = R_2 = OMe$ $R_3 =$ glucosyl
(11) $R_1 = OH$ $R_2 = H$ $R_3 =$ (2-O-xylosyl)glucosyl



(9) $R_1 = OTMS$ $R_2 = H$ $R_3 =$ TMS-(glucosyl)
(10) $R_1 = R_2 = OMe$ $R_3 =$ TMS-(glucosyl)
(12) $R_1 = OTMS$ $R_2 = H$ $R_3 =$ TMS-[(2-O-xylosyl)-glucosyl]

If an anthocyanin containing a disaccharide chain is investigated, a GLC peak with a higher retention time is obtained. For instance, the quinoline derivative 12, derived from cyanidin 3-O-[(2-O-xylosyl)glucoside] (11), exhibits a peak with a retention time of 9.2 min. It is of interest that, in these instances, the mass spectra provide useful information about the sequence of the sugar chain. In fact, in the spectrum of the derivative 12 (Fig. 5), in addition to the molecular ion peak at *m/e* 1299 and the two fragments at *m/e* 645 and 573 due to the loss of the disaccharide chain followed by the rearrangement of TMS and H radicals, strong peaks at *m/e* 349 [ion (c)] and 259 (349 — TMSOH) are present, which establishes the location of the xylose residue

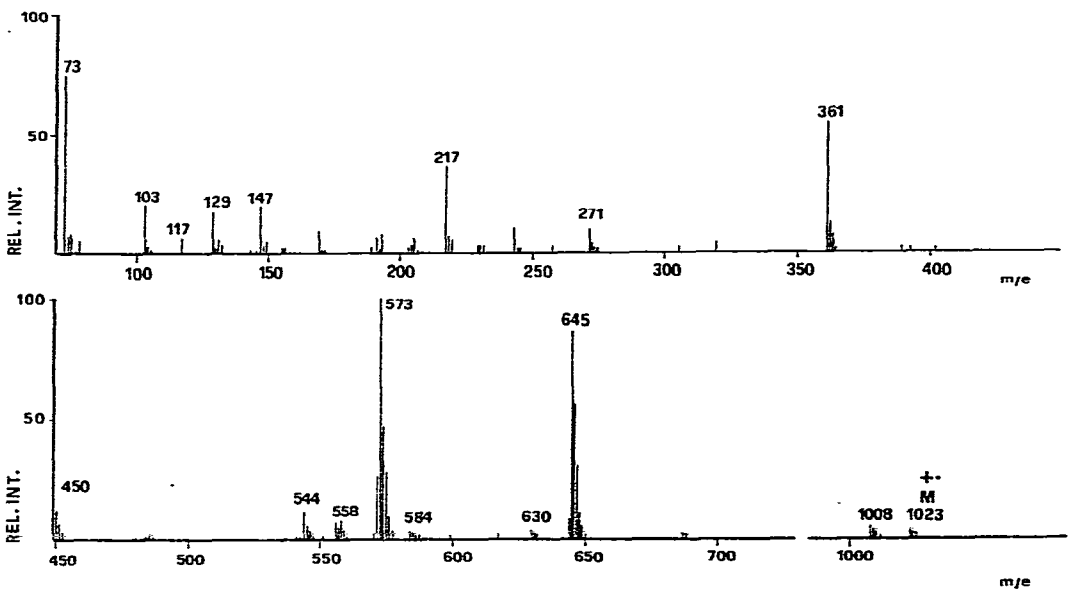


Fig. 3. Mass spectrum of derivative 9 (cyanidin 3-O-glucoside + HMDS + TMCS).

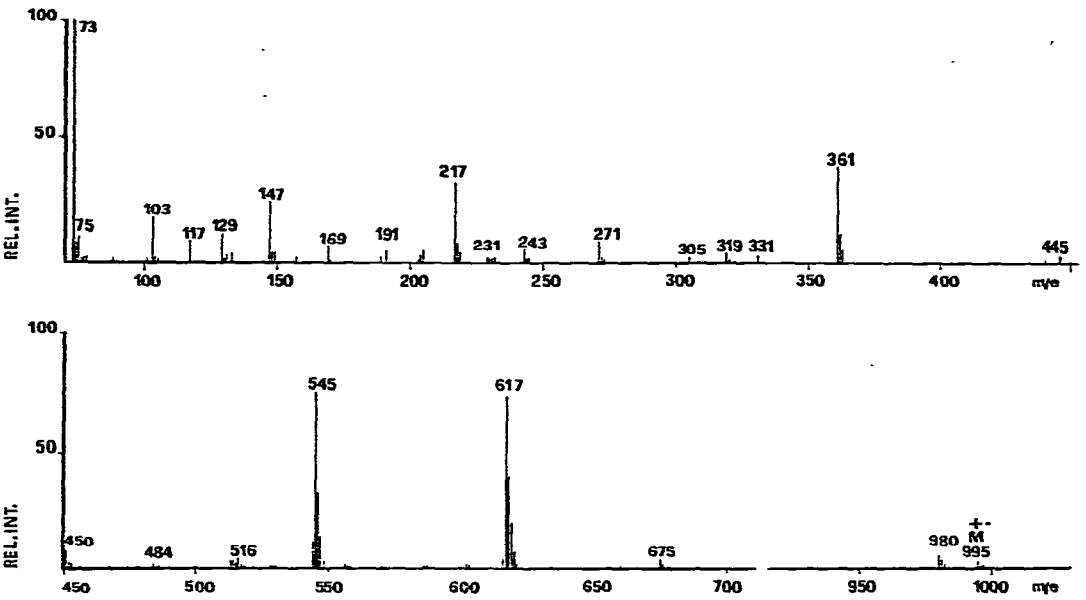


Fig. 4. Mass spectrum of derivative 10 (malvidin 3-O-glucoside + HMDS + TMCS).

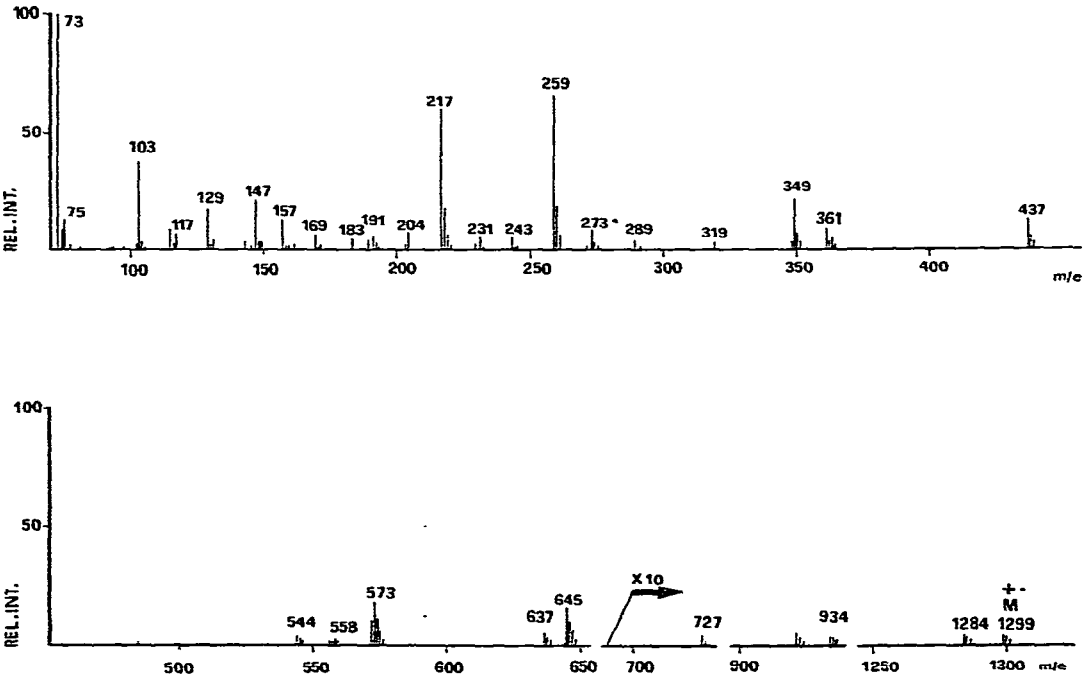
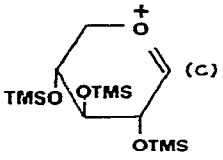


Fig. 5. Mass spectrum of derivative 12 [cyanidin 3-O-(2-O-xylosyl)glucoside + HMDS + TMCS].

at the end of the chain. The fragment ion at *m/e* 727 is due to the TMS-xylosyl-glucosyl residue from which the ion at *m/e* 637 originates through the loss of trimethyl-silanol (TMSOH).



An interesting phenomenon was observed when anthocyanins containing sugar chains on the hydroxyl function at position C-5 were investigated. In fact, all of the compounds examined exhibited two well separated GLC peaks. For instance, cyanidin 3,5-di-O-glucoside (13) gives rise (Fig. 6) to two peaks with retention times of 6.8 and 10.0 min, respectively. The mass spectrum of the former peak is identical with that obtained from cyanidin 3-O-glucoside (Fig. 3), whereas that of the latter (Fig. 7) is consistent with the expected structure 14. In fact, it contains a molecular ion peak

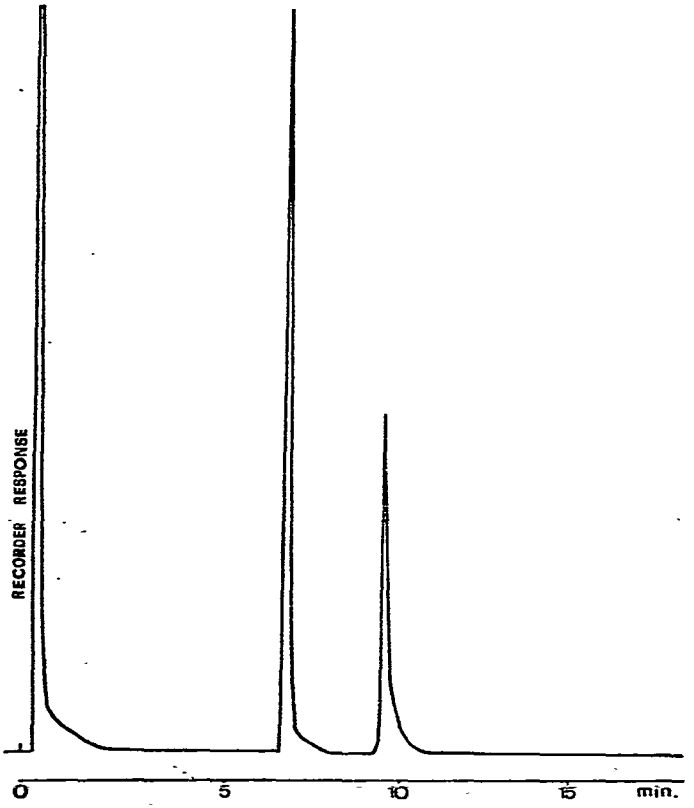


Fig. 6. Gas chromatogram of cyanidin 3,5-di-O-glucoside (13) after reaction with TMCS and HMDS.

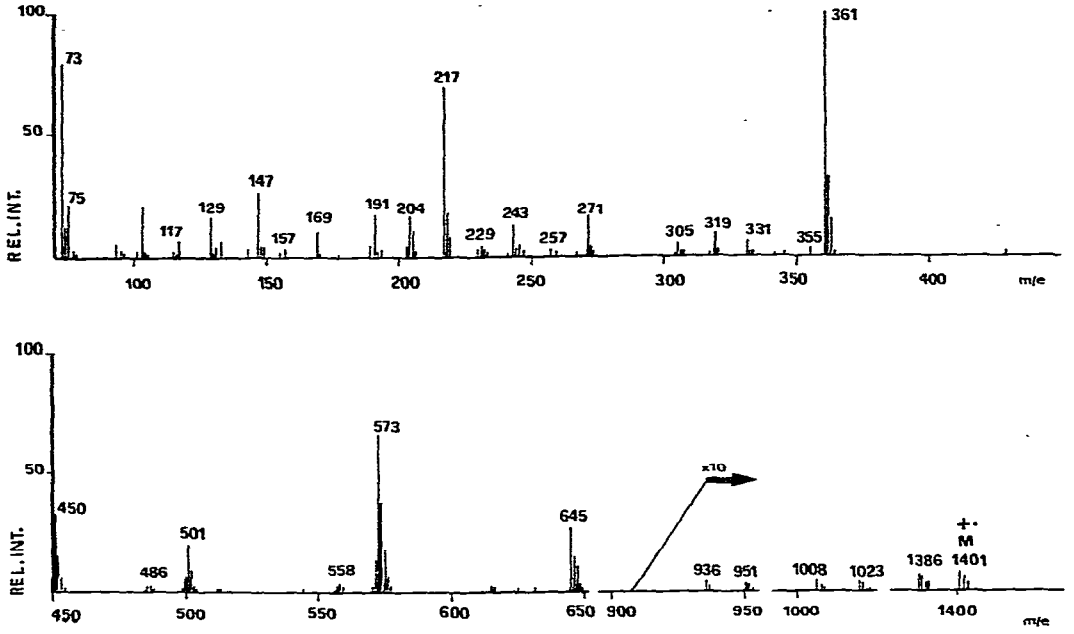
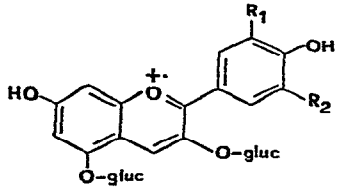
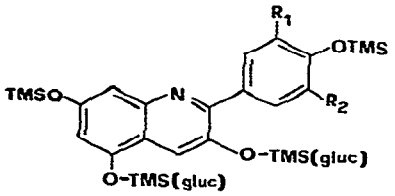


Fig. 7. Mass spectrum of derivative 14 (cyanidin-3,5-di-O-glucoside + HMDS + TMCS).

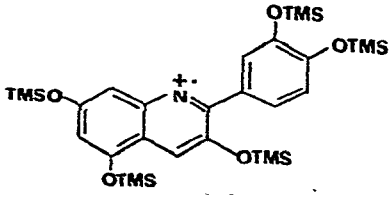
at *m/e* 1401, an $M^+ - CH_3$ peak at *m/e* 1386 and peaks at *m/e* 645 [ion (d)], 573 [ions (e) and (f)] and 501 [ion (g)], derived from the detachment of the TMS-glucosyl moieties followed by the usual rearrangement of H and/or TMS radicals.



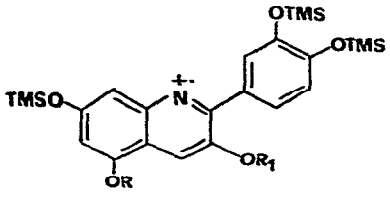
(13) $R_1 = OH$ $R_2 = H$
(15) $R_1 = OMe$ $R_2 = OMe$
(17) $R_1 = OH$ $R_2 = OMe$



(14) $R_1 = OTMS$ $R_2 = H$
(16) $R_1 = OMe$ $R_2 = OMe$
(18) $R_1 = OTMS$ $R_2 = OMe$



(d)



(e) $R = TMS$ $R_1 = H$
(f) $R = H$ $R_1 = TMS$
(g) $R = R_1 = H$

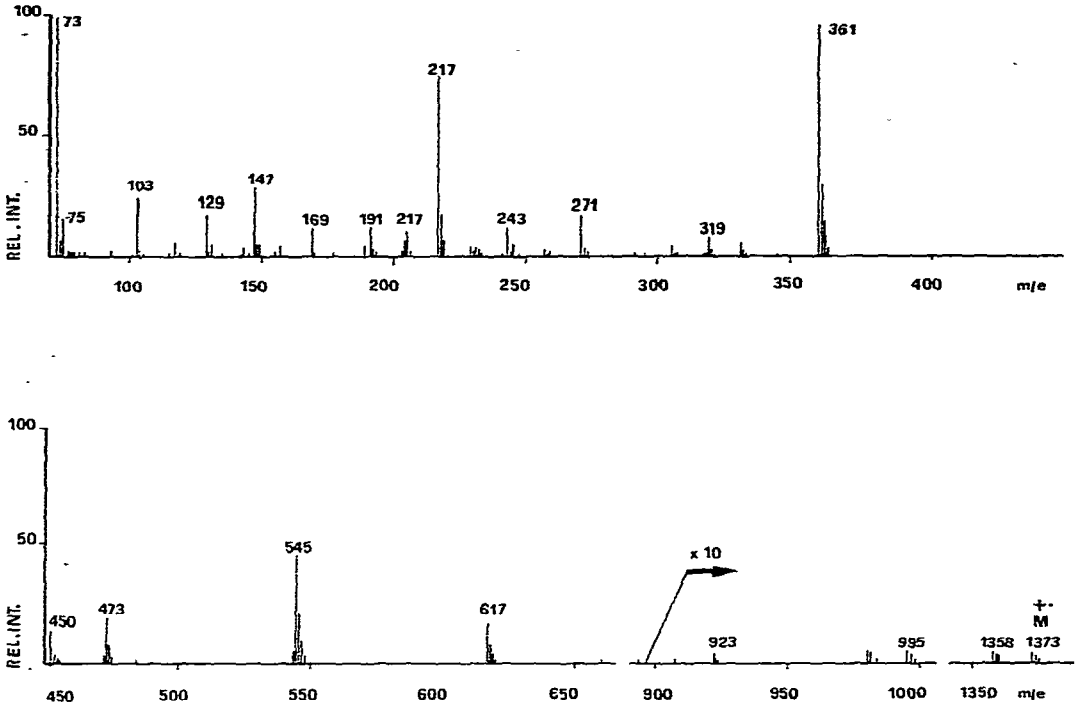


Fig. 8. Mass spectrum of derivative 16 (malvidin 3,5-di-O-glucoside + HMDS + TMCS).

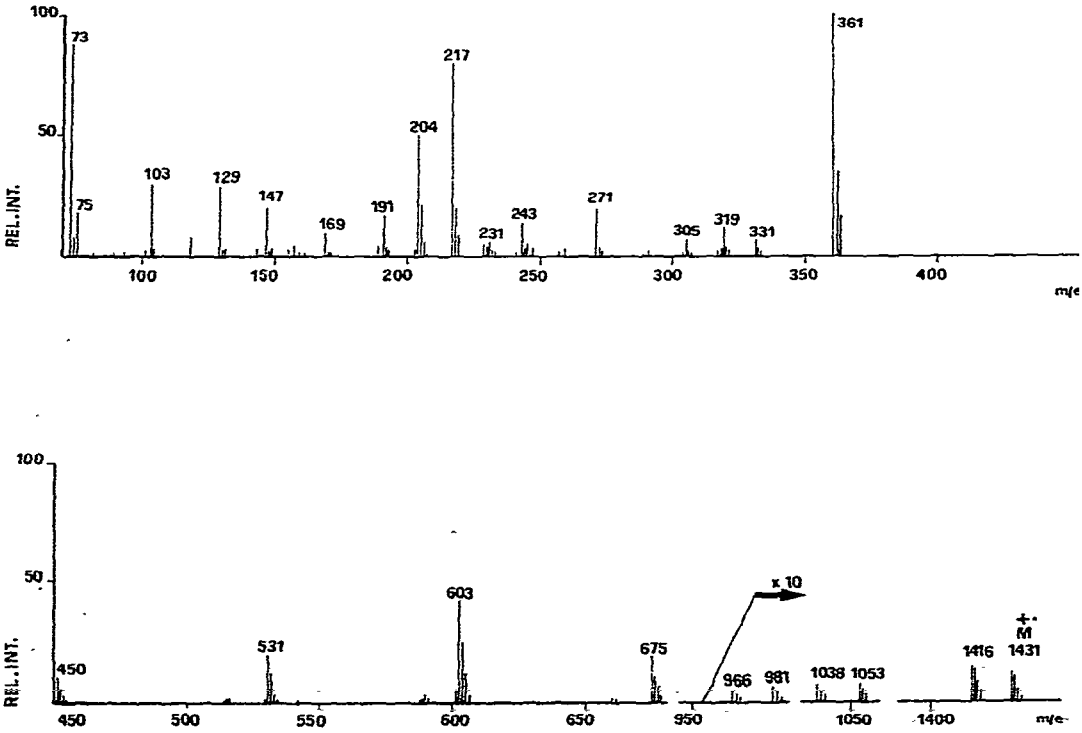


Fig. 9. Mass spectrum of derivative 18 (petunidin 3,5-di-O-glucoside + HMDS + TMCS).

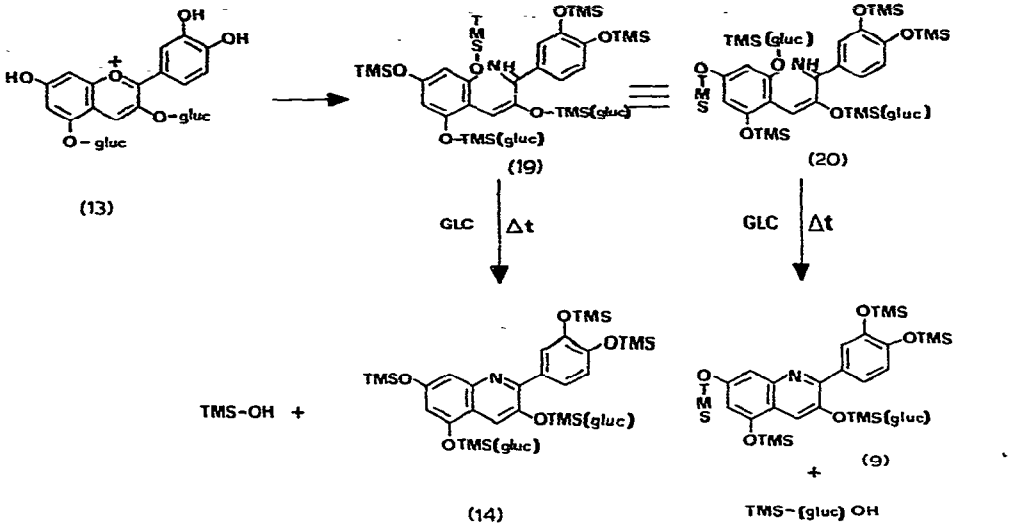
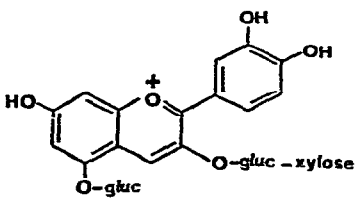


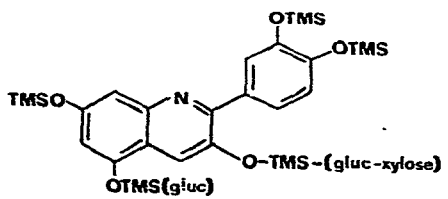
Fig. 10. Mechanism of formation of quinoline derivatives of cyanidin 3,5-di-O-glucoside (13).

Malvidin 3,5-di-O-glucoside (15) and petunidin 3,5-di-O-glucoside (17) display similar behaviour: the former generates two derivatives, 10 (retention time 7.9 min, mass spectrum shown in Fig. 4) and 16 (retention time 10.1 min, mass spectrum shown in Fig. 8), whereas 17 affords the quinolines 6 (retention time 7.5 min, mass spectrum shown in Fig. 2) and 18 (retention time 10.3 min, mass spectrum shown in Fig. 9).

This behaviour can readily be interpreted if the mechanism of formation of the quinoline derivatives is taken into consideration (Fig. 10). If the anthocyanin under investigation bears a group different from a methoxyl or a hydroxyl (*e.g.*, an O-glycosyl residue) at the C-5 position, there are two possibilities for the formation of the pyridine ring, depending on the elimination of a TMSOH or of a TMS-(glucose) group from the forms 19 and 20, with the consequent formation of the pairs of derivatives 9 and 14, 10 and 16, and 6 and 18, from 13, 15 and 17, respectively. The behaviour of cyanidin 3-(2-O-xylosylglucoside)-5-O-glucoside (21), which contains different sugar chains at positions C-3 and C-5, supports the correctness of this interpretation. On treatment with HMDS and TMCS and GLC analysis, 21 provides two GLC peaks with retention times of 9.2 and 11.8 min. The mass spectrum of the former derivative is identical with that obtained from the corresponding anthocyanin lacking the sugar chain in position C-5 (11, Fig. 5), whereas the latter (22) shows a mass spectrum (Fig. 11) containing the fragments of both of the different sugar chains.



(21)



(22)

It is of interest that, in all of the samples investigated, the GLC peak with the highest retention time is always the least intense, in accordance with a higher stability of form 20 in comparison with form 19, owing to the presence in the latter of a strong steric interaction between the two TMS-glucosyl residues. When the analysis was carried out at higher temperatures, an enhancement of the intensity of the minor peak was in fact observed.

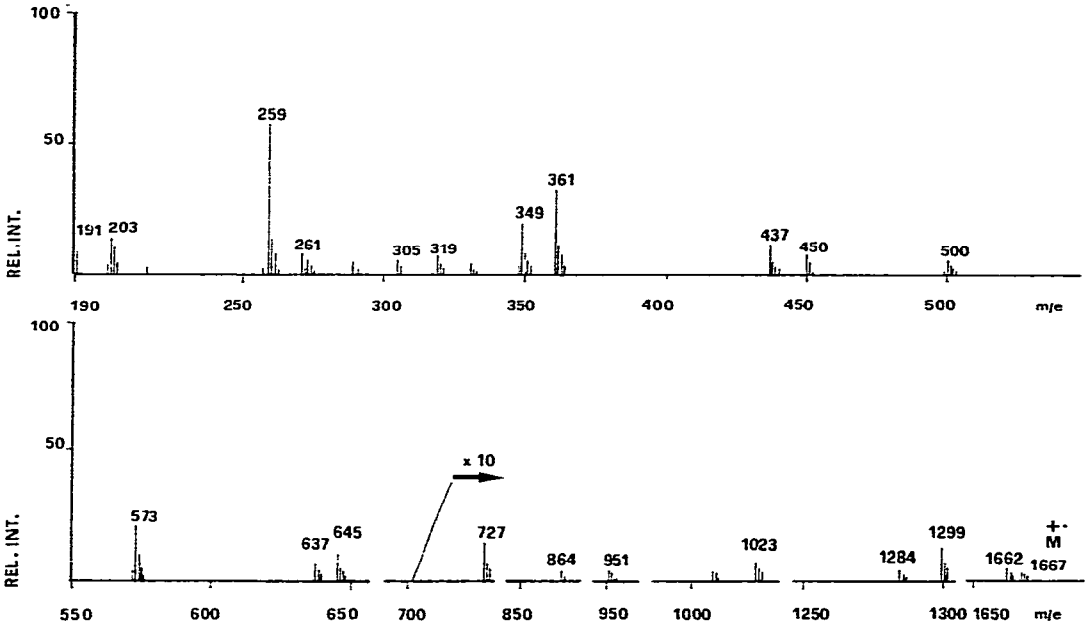


Fig. 11. Mass spectrum of derivative 22 [cyanidin 3-(2-O-xylosylglucoside)-5-O-glucoside + HMDS + TMCS].

CONCLUSION

The results indicate that GLC can be used satisfactorily for the analysis of these pharmacologically important natural substances. Some care has to be taken in the interpretation of the results as to whether the presence of sugar residues on the hydroxyl group at position C-5 of the anthocyanin skeleton is suspected; on the other hand, the presence of two peaks in the gas chromatogram of an anthocyanin indicates the presence of a glycosyl residue in position C-5.

At present, we are investigating the use of capillary columns and so far we have obtained results which indicate that this technique should be applicable to the analysis of very complex mixtures of anthocyanins.

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