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A MILD PROCEDURE FOR THE EXTRACTION AND FRACTIONATION OF ANTHOCYANIN, PROANTHOCYANIN AND OTHER POLYPHENOLS OF APPLE PEEL*

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Abstract—The filtrate obtained after a cold acetone extraction of fresh apple peel, separated into two phases upon addition of chloroform. The aqueous phase contained red pigments which were partially separated on Sephadex G-25 yielding two fractions. One-dimensional paper chromatography of these fractions revealed the presence of three anthocyanins and one polymeric proanthocyanin. Other phenols, also present in the aqueous and organic phases, were separated on two-dimensional paper chromatograms and identified.

INTRODUCTION

RECENTLY Webb[1] pointed out that milder isolation and purification methods were necessary to obtain the water-soluble plant pigments as nearly as possible in the forms in which they exist in the tissues. Generally, anthocyanins are extracted with alcoholic solvents containing some mineral acid, in order to isolate the pigments as the stable flavylium cation.[2]

In the extraction of acidic apple peel tissue (pH 3·0), mineral acids should not be necessary. In the present study, cold acetone was selected as the extractant for the peel polyphenols. This solvent has been shown previously to be an excellent solvent for phenols[3] and, additionally, serves the purpose of separating the enzymes from the substrates during the extraction procedure. This report describes the successful extraction and fractionation of anthocyanins and other polyphenols present in the apple peel.

RESULTS AND DISCUSSION

Essentially the procedure employed (for details—see Experimental section) was to prepare an acetone powder, and use the filtrate as the source of the apple peel polyphenols. Addition of one and a half volumes of chloroform to the aqueous acetone filtrate resulted in a two-phase separation. The aqueous layer, deep red in color, was shown to contain the anthocyanins. This fraction also contained most of the water-soluble glycosides, proanthocyanins and depsides. The lower organic layer did not contain anthocyanins nor proanthocyanins but did have some of the other phenols which were distributed in both phases.

The aqueous phase was concentrated and an aliquot subjected to gel-filtration on Sephadex G-25 (fine) according to the method of Somers,[4] and two main fractions were obtained which

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[1] A. D. WEBB, in *Phenolics Norm. Dis. Fruits Veg. Proc. Symp.*, 4th Norwood, Mass., p. 34 (1964).
[2] J. B. HARBORNE, in *Comparative Biochemistry of the Flavonoids*, p. 4, Academic Press, London (1967).
[3] SAID Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).
[4] T. C. SOMERS, *Nature* **209**, 368 (1966).

from their absorbance at 280 nm and 540 nm indicated separation between polymeric proanthocyanin (Fraction I; elution volume 10–40 ml) and monomeric flavan-3,4 diols and anthocyanins (Fraction II elution volume 40–110 ml). Both fractions I and II were subjected to paper chromatography on Whatman 3 MM paper.

Proanthocyanins

Fraction I was a polymeric phenol with a minimum molecular weight of 2000 estimated for complete exclusion from this particular gel.[4] Most of the fraction was immobile on paper chromatograms, developed in butanol–acetic acid–water (4:1:1) (BAW). The tan-colored band at the origin was extracted with hot water and, on treatment with butanol–hydrochloric acid (100:5) at 100°, gave mainly cyanidin. A smaller amount of an unidentified anthocyanidin was also produced which was not pelargonidin, previously reported as derived from proanthocyanin of fruit and leaves of apple.[5,6] The proanthocyanin of the peel therefore consists mainly of leucocyanidin.

Anthocyanins

One-dimensional chromatography on 3 MM paper of fraction II, using BAW as the developing solvent, resulted in the separation of II into one major red band and two minor pink bands. The major pigment was eluted with methanolic–HCl and from its spectra, R_f values and those of its acid hydrolysis products (Table 1), was identified as cyanidin-3-galactoside (idaein).[7]

TABLE 1. CHROMATOGRAPHIC AND SPECTRAL CHARACTERISTICS OF MAJOR APPLE PEEL PIGMENT AND PROANTHOCYANIN

	R_f		Spectra λ_{max} (nm)		Color*	
	BAW	Forestal	EtOH	$+AlCl_3$	1	2
Major red pigment	0·25	0·62	535	576		
Cyanidin	0·43	0·55	540	576		
Unknown aglycon	0·42	0·55	540	—		
Unknown sugar	0·22	—	—	—	Brown	None
Galactose	0·22	—	—	—	Brown	None
Glucose	0·24	—	—	—	Brown	Yellow-brown
Proanthocyanin band	0·00	Decomp.	280	—		
Proanthocyanin after BuOH–HCl	0·42	0·55	554†	—		

* 1—aniline phthalate dip. 2—glucostat reagent.
† Difference in cyanidin max is due to butanol–HCl replacing ethanol.

After elution and rechromatography in other solvents the minor pigments were still not separable from larger amounts of quercetin-3-glycosides. A recent report of Sun and Francis[8] indicates that they may be cyanidin-3-arabinoside and 7-arabinoside.

[5] S. ITO and M. A. JOSLYN, *J. Food Sci.* **30**, 44 (1965).
[6] S. LEWAK, B. PLISZKA and E. EICHELBERGER, *Acta. Soc. Botan. Pol.* **36**, 251 (1967).
[7] C. E. SANDO, *J. Biol. Chem.* **117**, 45 (1937).
[8] BONNIE H. SUN and F. J. FRANCIS, *J. Food Sci.* **32**, 647 (1967).

Other Phenols

(−)Epicatechin, (+)catechin, chlorogenic acid and quercetin-3-glycosides were tentatively identified in the aqueous and organic phases. These substances are well-known constituents of apple peel.

This method of extraction and fractionation using cold acetone has been shown to be a useful technique in that most of the classes of the known apple polyphenols were separated from the extract and many could be quickly identified by chromatographic and spectrophotometric methods. The use of Sephadex G-25 was promising as a means of isolation of polymeric proanthocyanin which could be separated from the monomeric and dimeric flavans. The organic phase ($CHCl_3$–acetone) did not contain anthocyanins or proanthocyanins, but did reveal a substance tentatively identified as quercetin. This suggested that either a slight degree of acid hydrolysis occurred during the extraction or that free quercetin is present in apple peel.

EXPERIMENTAL

Acetone Powder

100 g pared McIntosh apple peel was vacuum infiltrated with 350 ml acetone, pre-cooled to −20°. The extracted peel was then passed through once between knurled stainless-steel rollers and the resulting powder collected in pre-cooled acetone and filtered. After washing the powder with cold acetone the filtrate and washings were combined. The dried powder (conc. H_2SO_4) was stored at 0° *in vacuo* for further use.

Fractionation of the Acetone Extract

The acetone extract was treated with $CHCl_3$ (2:3 v/v) and the top aqueous layer carefully separated. This layer was concentrated *in vacuo* over H_2SO_4 and the $CHCl_3$ layer evaporated to small volume suitable for two-dimensional chromatography.

Gel-Filtration

The concentrated aqueous layer was taken up in a small volume of a 60% ethanol solution containing 0·1% HCl and portions (1 or 2 ml) subjected to gel-filtration on Sephadex G-25 (fine) according to the method of Somers.[4]

Chromatography

Whatman No. 1 paper was used for two-dimensional chromatography and 3 MM paper for uni-dimensional chromatography. Ascending chromatography was used and chromatograms were developed in the following solvent systems as needed: BAW = 1-butanol–acetic acid–water (4:1:1); Forestal = acetic acid–conc. HCl–water (30:3:10); 6% glacial acetic acid.

The polyphenols were detected by using ferric-chloride–ferricyanide,[9] diazotized sulfanilic acid,[10] and 2% vanillin in ethanol mixed with an equal volume of conc. HCl. Sugars were detected in acid hydrolysates after chromatography in BAW with an aniline phthalate dip.[11] Galactose was distinguished from glucose by using the Worthington glucostat reagent as a spray.

Spectrophotometry

U.v. spectra of eluted compounds and authentic substances were obtained using a Bausch and Lomb spectronic 502 recording spectrophotometer in either 95% ethanol or butanol–HCl (100:5). Spectral shifts were obtained with a few drops of $AlCl_3$[12] added to the cuvette.

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