Journal of Chromatography A, 657 (1993) 445–449
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 578

Short Communication

Purification of fresh cassava root polyphenols by solid-phase extraction with Amberlite XAD-8 resin

Fernando Lalaguna

Unidad de Tecnología Nuclear, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020-A (Venezuela)

(First received April 20th, 1993; revised manuscript received September 21st, 1993)

ABSTRACT

To obtain the polyphenolic fraction of fresh cassava root in a form amenable to analysis by thin-layer chromatography (TLC) and absorption spectroscopy, aqueous concentrates of delipidated extracts were loaded on 0.3 g (dry mass) of Amberlite XAD-8 resin, non-polyphenolics were washed off with water and polyphenols eluted with methanol–water (7:3, v/v). The resolution and purity of polyphenols were ascertained with four TLC systems. In addition, the system exhibits complete recovery and is reproducible, and the resin withstands repeated use without lost of performance, which makes it an efficient method.

INTRODUCTION

Extracts of polymeric plant polyphenols have been processed by solid-phase extraction with C_{-18} cartridges to allow their analysis by TLC and other methods [1]. However, although several aspects of the macroreticular resins of the Amberlite XAD series have been investigated, including their chromatographic properties [2–4], use for the separation of phenols [5] and other compounds [6,7], and for the isolation of polyphenols [8,9], their use to purify polyphenolic plant extracts by solid-phase extraction has not been reported.

Pietrzyk and Chu [6] pointed out that for stripping applications the stronger XAD adsorbent might be preferred. In accordance with this, McRae *et al.* [4] found that Amberlite XAD-8, a polar acrylic ester resin, was useful for the separation of polar solutes from aqueous extracts, while Amberlite XAD-2, a non-polar copolymer of styrene–divinylbenzene, was not satisfactory. On the other hand, Vian et al. [9] did not find the acrylic ester-type Amberlite suitable for the retention of carbohydrates. This indicates the applicability of this resin to deprive adsorbable polyphenols of sugars.

Polyphenols containing phenylpropanoid, coumarin and flavonoid structures have been reported in the roots of cassava (*Manihot esculenta*, Crantz) and the flour prepared from them [10–13]. Coumarins are by far the main component; the other polyphenols, *e.g.* catechins, are minor, and proven to be present in the experimentally injured root's parenchyma [11–13]. These polyphenols have not been assayed on Amberlite XAD resins.

For the continuation of our studies on cassava root discoloration [14,15] a method to purify the polyphenols extracted from cassava root that would permit their analysis by TLC, UV–Vis spectroscopy and other methods was needed. A

0021-9673/93/$06.00

successful process using Amberlite XAD-8 was developed and is reported here.

EXPERIMENTAL

Chemicals

The solvents and reagents used were of analytical grade, and from either Fisher Scientific (Fair Lawn, NJ, USA) of Merck (Darmstadt, Germany). Polyphenols and other standards were from Sigma (St. Louis, MO, USA). Amberlite XAD-8 (Rohm & Haas) was also obtained from Sigma.

Resin preparation

Amberlite XAD-8 was thoroughly washed before use as follows: 10 g of resin were poured into 300 ml of 0.1 *M* sodium hydroxide in a filtering flask, degassed under the vacuum of a water jet pump, and stirred. Excess sodium hydroxide was washed off with 300 ml of water, 300 ml of 0.1 *M* hydrochloric acid added and the resin shaken. After discarding the acid the resin was washed with water in a büchner funnel until the filtrate was neutral (universal indicator paper). Excess water was removed with ethanol and the resin was extracted in a Soxhlet apparatus for 7 h with the following solvents, leaving the resin immersed in the same solvent overnight: toluene, acetone, ethanol and methanol. The resin was rinsed with the new solvent whenever there was a change of solvent.

The washed resin was ground when dry with a porcelain mortar and pestle, and the 0.250 mm fraction (dry) collected. The fines of the XAD-8 resin had to be removed with organic solvents [methanol–water or acetone–water, 7:3 (v/v) would do] since water was found to be unable to eliminate all the fines. Keeping the unused resin in organic solvents is not advisable.

Column packing and operation

For the processing of crude cassava root extracts, an Econo-Column (Bio-Rad, Richmond, CA, USA) glass column 10 cm × 0.7 cm I.D., with a polypropylene tip and polyethylene bed support, was packed with a water suspension of the XAD-8 resin. Afterwards, the resin was washed with methanol–water (7:3) and reversed to water. A final bed volume of 1 ml (approximately 0.3 g of dry XAD-8 resin) was used. A Pasteur pipette plugged with glass wool was substituted for the Econo-Column for the purification of TLC-resolved polyphenols to be analysed by UV–Vis spectroscopy, otherwise the organic parts of the Econo-Column and tubing contribute interfering substances.

Prior to the application of the sample, the column was rinsed with 10 ml of methanol–water (7:3) followed by 10 ml of water, then the sample was loaded, the non-polyphenolic material was washed out with 10 ml of water, and the polyphenols eluted with 10 ml of methanol–water (7:3). Flow-rates between 0.2 and 1 ml/min were used. The methanolic eluates were taken to dryness under a stream of nitrogen at 35°C, and the residue dissolved in 1 ml of methanol–water (7:3).

When performing the above procedure, the washings and eluates were collected as 1-ml fractions for further analysis.

Extraction of cassava

Fresh cassava root cv. Algodona (a sweet, white cultivar with small roots) of 10 months was purchased from a producer. Samples of fresh roots that included only the uninjured parenchymatous cylinder were extracted as previously reported [14] followed by extraction with two 100-ml portions of acetone–water (7:3) in an Omni-Mixer (Omni, Waterbury, CT, USA). The acetone extract was mixed with the polar phase of the previous extraction, and ten-fold concentrated under vacuum in a rotary evaporator at 35°C. Any deposit that formed was removed by centrifugation at low speed. Aliquots of the aqueous extracts, concentrated as indicated above, which had a pH of about 5 and a dry mass of the order of 7 mg, were percolated down with XAD-8 resin column.

TLC and spectroscopy

To check the nature of the compounds in the fractions collected from the XAD-8 resin column during the development of the solid-phase extraction system, these fractions were subjected to TLC on microcrystalline cellulose with butanol–acetic acid–water (4:1:5, v/v/v) [16] as solvent

system, and on silica gel G developing with both toluene–ethyl formate–formic acid (5:4:1, v/v/v) or ethyl acetate–methyl ethyl ketone–formic acid–water (5:3:1:1, v/v/v) [17]. The layers were visualized with 366-nm UV light, before and after exposure to ammonia vapours, and by spraying with 1-naphthol (for sugars), ninhydrin and arabinose–aniline (for organic acids [18]) reagents.

To verify that the purification process had rendered the polyphenols susceptible to analysis by TLC and UV–Vis spectroscopy, aliquots of the polyphenolic fraction were streaked on 0.5-mm-thick preparative silica gel G (Merck) layers and developed three times, once for 15 cm and twice for 12 cm, with the solvent system chloroform–methanol–acetic acid–water (170:25:25:6, v/v) [14]. The bands were localized by exposing the plates to 366-nm UV light, their positions marked, the silica gel scraped off the plates, the compounds extracted with three 1-ml portions of ethanol–formic acid–water (8:1:1, v/v), taken to dryness, and dissolved in methanol. The procedures given by Markham [19] were followed to obtain the UV–Vis spectra of the eluted bands. A Perkin-Elmer (Norwalk, CT, USA) Model Lambda 3B dual spectrophotometer was used.

Analytical methods

For total phenols a method using Folin and Ciocalteau reagent was used [20]. For catechins the vanillin methods of Price *et al.* [21] and of Butler *et al.* [22] were followed. Column elution was monitored by manually measuring the absorbance of the fractions at several wavelengths and/or by chemical analysis [20,23–26].

RESULTS AND DISCUSSION

In order to further our studies on cassava root discoloration, the analysis of the polyphenols allegedly responsible for this process [11–13] became essential. The analysis of crude polyphenol extracts was hampered by the high concentration of other compounds, so purification of the extracts was required.

Standards of the kind of compounds reported in cassava root [10–13,15] or similar tissues were assayed in the Amberlite XAD-8 solid-phase extraction system devised, to determine the feasibility of this system for the purification of cassava extracts. The results for some of these compounds are given in Table I. Other substances assayed (data not shown) exhibited behaviours similar to those of their chemical analogues in Table I, among them the organic acids citric, succinic, shikimic and quinic, the amino acids tryptophan and histidine, and the coumarins esculetin and esculin. Rutin and (+)-catechin were eluted with methanol–water (7:3), while chlorogenic acid required acetone–water (7:3).

The results show that the interaction with Amberlite XAD-8 of the non-polyphenolics tested is weak enough to permit their complete displacement with water, including aromatic amino acids, even though, like polyphenols, they contain a benzene ring.

On the contrary, none of the polyphenols assayed was eluted by water; they required either methanol–water (7:3) or acetone–water (7:3), depending on their structure. Scopoletin, esculin and (+)-catechin, which are reportedly found in cassava root [11–13], where all eluted in the same fraction. These polyphenols are completely recovered from the resin, *e.g.* scopoletin, the main polyphenol in cassava [11,13], was 98 ± 6% (standard deviation, $n = 3$) recovered, on average, when loading the system with 2–50 μg of scopoletin. The behaviour of these standards in isolation is maintained when in admixture, as demonstrated by the separation of a mixture of tyrosine, rutin and quercetin (Table I). The results indicate that the system is capable of separating the polyphenols found in cassava root from other widely distributed substances, including intermediates of polyphenol metabolism.

The data for the elution of the cassava root extracts are included in Table I, where it can be seen that most of the acids and carbohydrates, and all the ninhydrin-reactive substances, were eluted with water. Methanol–water (7:3) eluted one peak of material measurable with Folin and Ciocalteau reagent [20]. This material gave a feeble response with reactives for total sugars and acidic groups. The separation of the polyphenols from other compounds was verified with four TLC systems, which showed that methanol–

TABLE I

ELUTION OF STANDARD COMPOUNDS AND CASSAVA ROOT EXTRACT FROM AMBERLITE XAD-8 RESIN

A 1-ml volume of wet, 0.250 mm Amberlite XAD-8, flow-rate 0.2–1 ml/min, 1-ml fractions, gravity flow.

Sample	Absorbance																	
	Eluent/fraction number																	
	Water						Methanol–water (7:3, v/v)						Acetone–water (7:3, v/v)					
	1	2	3	4	5	10	11	12	13	14	15	20	21	22	23	24	25	30
Glucose[a] (260 nm)	1.52	1.84	0.13	0.16	0.18	0.00												
Malic acid[b] (470 nm)	0.13	1.58	0.26	0.06	0.07	0.00												
Ascorbic acid[c] (297 nm)	0.95	2.13	0.07	0.02	0.02	0.00												
Tyrosine[d] (280 nm)	0.06	0.66	0.15	0.05	0.05	0.00												
Scopoletin[e] (280 nm)	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.17	0.36	0.16	0.04	0.00						
Caffeic acid[f] (470 nm)	0.00	–	–	–	–	0.00	0.00	–	–	–	–	0.00	0.34	0.21	0.00	–	–	0.00
Quercetin[g] (350 nm)	0.00	–	–	–	–	0.00	0.00	0.03	0.06	0.08	0.09	0.11	0.15	1.01	0.47	0.07	0.01	0.00
Mixture of[h] standards (280/350 nm)	0.02	0.03	0.61	0.16	0.00	0.00	0.00	0.67	0.58	0.31	0.23	0.14	0.19	1.29	0.48	0.09	0.02	0.00
Cassava extract																		
(470 nm)[i]	0.00	0.86	1.13	0.08	0.00	0.00	0.00	0.01	0.00	0.08	0.01	0.01	0.00	–	–	–	–	0.00
(560 nm)[j]	0.01	1.69	2.53	0.18	0.05	0.01	0.01	0.04	0.01	0.00	0.00	0.01	0.01	0.10	0.00	–	–	0.00
(570 nm)[k]	0.01	1.11	1.14	0.06	0.03	0.03	0.03	0.03	0.04	0.04	0.03	0.05	0.01	–	–	–	–	0.00
(725 nm)[l]	0.01	0.27	0.31	0.14	0.12	0.04	0.15	0.41	0.11	0.05	0.02	0.00	0.01	0.03	0.02	0.01	0.01	0.00

[a] A 0.25-ml aliquot of 1 mg/ml aqueous glucose, measured by the method of Agranoff *et al.* [24].
[b] A 0.25-ml aliquot of 1 mg/ml aqueous malic acid, fractions titrated with sodium ortho-nitrophenol [25].
[c] A 0.20-ml aliquot of 1 mg/ml aqueous ascorbic acid, direct absorbance reading.
[d] A 0.25-ml aliquot of 0.4 mg/ml aqueous tyrosine, direct absorbance reading.
[e] A 0.25-ml aliquot of scopoletin aqueous saturated solution, direct absorbance reading.
[f] A 0.10-ml aliquot of caffeic acid aqueous saturated solution, fractions titrated with sodium ortho-nitrophenol [25].
[g] A 12.5-μl aliquot of 4 mg/ml ethanolic quercetin, loaded in 1 ml of water, direct absorbance reading.
[h] A 0.25-ml aliquot of 0.4 mg/ml aqueous tyrosine, plus 0.25 ml of rutin aqueous saturated solution, plus 12.5 μl of 4 mg/ml ethanolic quercetin; water eluates direct reading at 280 nm, others at 350 mm.
[i] Fractions titrated with sodium ortho-nitrophenol [25].
[j] Fractions analysed for sugars [23].
[k] Fractions reacted with ninhydrin [26].
[l] Fractions analysed for Folin and Ciocalteau reactive substances [20].

water (7:3) eluates did not produce any band for free sugars, organic acids (arabinose–aniline visualization [18]) or amino group-containing compounds, but gave bands positive for polyphenols. Conversely, water eluates failed to produce bands of polyphenols, but bands were visualized for all the other components. This shows that the methanol–water (7:3) eluates contain the polyphenolic fraction in pure form.

The recovery for cassava extracts was 91.2 ± 7% (S.D., $n = 7$) on average, through a range of load of 63–188 μg (as scopoletin); 20–30% of the loaded material was adsorbed depending on the root extracted.

Overloading, or fouling with repeated use, of the XAD-8 resin with cassava extracts could cause acetone–water (7:3) to remove a small amount of material from the resin (see Table I).

TLC analysis showed that this material was similar to that eluted by methanol–water (7:3).

After their purification, the polyphenols were neatly resolvable by TLC. A maximum of eight intense plus two weak bands were observed. The compound in the main band, after its extraction and cleaning with the all-glass system described, gave UV–Vis spectra [19] equivalent to those for scopoletin. Processing cassava extracts with the solid phase extraction system reported permitted verification of the absence of polyphenols with a flavonoid nucleus in extracts prepared from sound root's parenchyma, excluding any injured surface; three cassava cultivars were used. In contrast, this type of polyphenol has been detected with histochemical techniques [12] and measured in extracts prepared from experimentally injured root [11–13].

The Amberlite XAD-8 solid-phase extraction procedure reported has proven to be reproducible through 2 years of use, with both standards and different cassava extracts, which together with the above results —effective purification and high recovery, and the stability, sustained usability and economy of the resin— makes it an efficient method to render cassava polyphenol extracts susceptible to further analysis. The performance of the method suggests that it could be used successfully on other plant extracts.

ACKNOWLEDGEMENT

This work was partially financed by CONICIT Project S1-1909.

REFERENCES

1 H.A. Stafford and H.H. Lester, *Plant Physiol.*, 66 (1980) 1085.
2 D.J. Pietrzyk, and C. Chu, *Anal. Chem.*, 49 (1977) 757.
3 E.M. Thurman, R.L. Malcolm and G.R. Aiken, *Anal. Chem.*, 50 (1978) 775.
4 T.G. McRae, R.P. Gregson and R.J. Quinn, *J. Chromatogr. Sci.*, 20 (1982) 475.
5 J.S. Fritz and R.B. Willis, *J. Chromatogr.*, 79 (1973) 107.
6 D.J. Pietrzyk and C. Chu, *Anal. Chem.*, 49 (1977) 860.
7 E.P. Kroeff and D.J. Pietrzyk, *Anal. Chem.*, 50 (1978) 502.
8 E.M. Thurman and R.L. Malcolm, *Environ. Sci. Technol.*, 15 (1981) 463.
9 A. Vian, E. Castro and J.J. Rodríguez, *Separation Sci. Technol.*, 20 (1985) 481.
10 S. Sefa-Dedeh and V.F. Rasper, *Cereal Chem.*, 54 (1977) 746.
11 J.E. Rickard, *Trop. Sci.*, 23 (1981) 235.
12 J.E. Rickard and P.B. Gahan, *Ann. Bot.*, 52 (1983) 811.
13 Y. Tanaka, E.S. Data, S. Hirose, T. Taniguchi and I. Uritani, *Agric. Biol. Chem.*, 47 (1983) 693.
14 F. Lalaguna and M. Agudo, *J. Am. Oil Chem. Soc.*, 65 (1988) 1808.
15 F. Lalaguna and M. Agudo, *Phytochemistry*, 28 (1989) 2059.
16 J.B. Harbone, *Phytochemical Methods*, Chapman & Hall, London, 1973, p. 33.
17 E. Stahl and P.J. Schorn, *Hoppe-Seyler's Z. Physiol. Chem.*, 325 (1961) 263.
18 M. Bourzeix, J. Guitraud and F. Champagnol, *J. Chromatogr.*, 50 (1979) 83.
19 K.R. Markham, *Techniques of Flavonoid Identification*, Academic Press, New York, 1982, p. 36.
20 T. Swain and W.E. Hillis, *J. Sci. Food Agric.*, 10 (1959) 63.
21 M.L. Price, S. Van Scoyoc and L.G. Butler, *J. Agric. Food Chem.*, 26 (1978) 1214.
22 L.G. Butler, M.L. Price and J.E. Brotherton, *J. Agric. Food Chem.*, 30 (1982) 1087.
23 A.W. Devor, *J. Am. Chem. Soc.*, 72 (1950) 2008.
24 B.W. Agranoff, R.M. Bradley and R.O. Brady, *J. Biol. Chem.*, 23 (1958) 1077.
25 L. Kesner and E. Muntwyler, *Anal. Chem.*, 38 (1966) 1164.
26 S. Moore and W.H. Stein, *J. Biol. Chem.*, 211 (1954) 907.