

STUDIES ON ADSORBENTS FOR THE PREPARATION OF CAROTENOIDS AND CHLOROPHYLLS CONCENTRATES FROM BERSEEM (ALFALFA)

MAHMOOD AHMAD SHAH and MANZOOR ELAHI

P.C.S.I.R. Laboratories, Lahore

(Received May 22, 1969)

The suitability of various locally available adsorbents for the preparation of carotene and chlorophylls concentrates from alfalfa has been studied. Sugar and starch have given the most promising results for the separation of carotenoids and chlorophylls. Regeneration of some of the adsorbents by solvent washing and drying at 85°C for two hours, for re-use, has also been investigated. Both sugar and starch may be used six times after regeneration before packing the columns.

Previously^{1,2} carotenoid content of various green plants and effects of dehydration and storage on the carotenoid content of berseem have been reported. Carotenoids and chlorophylls apart from being very useful natural food colours, are compounds of biological, medicinal and industrial importance. These compounds occur in substantial amounts in alfalfa, which is also very rich source of proteins. This highly nutritive plant may be processed to produce β -carotene (provitamin A) xanthophylls, chlorophylls and proteins. Proteins and carotenes thus isolated could be usefully utilized for vitamins A fortification and protein supplementation of foods to help in combating malnutrition in the country.

Carotene concentrates from chlorophyll—containing plant tissues can be prepared by different procedures. Recently adsorption chromatography³⁻⁷ has been used for the separation of carotenoids and chlorophylls. The aim of the present investigations is to study the suitability of locally available adsorbents for the separation of plant (berseem) pigments. The suitability of any adsorbent would depend upon its capacity to separate the carotenoids and chlorophylls, easy availability, low cost and easy regeneration for re-use.

No attempt has been made here to identify the different carotenoids and chlorophylls, and these have been estimated as β -carotene and total chlorophyll respectively. Treatment of the residue for the isolation of proteins would be dealt with in a separate communication.

Experimental

Chemicals.—Solvents used were of commercial grade. Talc and bauxite were indigenous materials. Calcium phosphates were prepared in these laboratories. Sugar, starch and sodium carbonate were obtained as commercial grade products. All adsorbents except alumina were

passed through 36 mesh sieve and the fraction retained on 100 mesh was used. Alumina (Merck) was deactivated and used as standard. Unicam 600 was used for spectrophotometric estimation.

Procedure.—Berseem leaves were collected in the month of December and mixed well to get a composite sample. Extraction of pigments was done by the following procedures:

Procedure 1: It was essentially Kemmerer and Fraps⁸ procedure which has been described earlier.¹

Procedure 2: Berseem leaves (10 g) were homogenized in 1:1 mixture of light petroleum (60–80°C) and acetone for five minutes in a Waring blender, in the presence of $MgCO_3$ (0.1 g), allowed to stand and decanted. The residue was again blended with solvent mixture for two minutes, filtered under suction, and the residue washed with the solvent mixture until the latter was colourless. Acetone was removed by washing with water, petroleum ether layer dried (Na_2SO_4) and made to a definite volume (500 ml) 10 ml of this extract was chromatographed on columns packed with different adsorbents. Carotenoids were eluted with light petroleum, eluate made to a definite volume and estimated spectrophotometrically in terms of β -carotene. The chlorophylls were eluted with 30:70 mixture of acetone and light petroleum. The solvents were removed under reduced pressure and the chlorophylls estimated according to the method of Comar and Zscheile.⁹ The results are recorded in Table 1. The concentrates are prepared by removing the solvents from the solutions of carotenoids and chlorophylls as obtained above, under reduced pressure.

Recovery of pure β -carotene by column chromatography using different adsorbents.

2 ml of standard solution of purified β -carotene (5 mg %) in light petroleum was chromatographed

on columns packed with different adsorbents (10 g each) and β -carotene eluted with light petroleum. The eluate made to a definite volume and estimated spectrophotometrically. The results are given in Table 2.

Amounts of various adsorbents required to separate the carotenoids and chlorophylls from the extract in Flask Method.

Adsorbent was added to a definite volume (10 ml) of the plant extract in a flask until, upon settling, no green colour was apparent in the supernatant liquid. The latter was removed, residue washed with light petroleum until colourless, washings combined, made to a definite volume and estimated spectrophotometrically. Chlorophylls were removed from the adsorbent by shaking with 30% acetone in light petroleum and estimated as mentioned before. The results are recorded in Table 3.

Regeneration of the Adsorbents

1. *Solvent Washing*.—The columns were washed with sufficient amount of light petroleum to remove acetone before introduction of the fresh extract.

2. *Drying*.—The adsorbent was extruded from the column and dried at 85°C for two hours in an oven after eluting chlorophylls. The column was re-packed with the dried adsorbent and used again.

Regeneration in both the methods was continued until carotenoids and chlorophylls tend to come together. The results are shown in Table 4.

Discussion

It will be observed in Table 1 that except talc and calcium carbonate all other adsorbents give almost 100% recovery of purified β -carotene taking alumina as the standard. It has been noted that the rate of flow of solvent through talc and calcium carbonate is very slow and the loss of β -carotene during chromatography may be attributed to its adsorption and oxidation due to prolonged exposure to air. The carotenoid bands were not conspicuous on bauxite on account of its yellowish colour.

Table 2 includes the carotenoid and chlorophyll contents of berseem as determined by two procedures. In the first, chlorophylls, carotenoid esters and alkali-labile carotenoids are removed by alkali treatment. Thus this extract contains total carotenoids except the above-mentioned ones. Carotenes are obtained, by chromatography of this extract on alumina, in the eluate and the fraction adsorbed represents the xanthophylls. It is obvious from table 2 that in spite of the variation in their moisture contents, alumina, bauxite and calcium phosphates give almost similar amounts of carotenes which shows their equivalent adsorptive capacity. Adsorbents like talc and calcium carbonate give lower results. The difference in the carotenoid contents before and after chromatography on sugar and starch, is very small which signifies that most of the xanthophylls are also eluted along with carotenes. In the second procedure, the carotenoid contents after chromatography are higher than those obtained in the first procedure. This increase may be attributed to the carotenoid esters which being less polar than other xanthophylls, are eluted along with carotenes.

TABLE 1.—CAROTENOID AND CHLOROPHYLL CONTENTS OF ALFALFA (BERSEEM) LEAVES AS DETERMINED BY CHROMATOGRAPHY USING DIFFERENT ADSORBENTS.

Adsorbents	Moisture %	Carotenoid Content (mg/kg)		Chlorophylls (mg/kg) total	a	b	a/b
		Procedure I	Procedure II				
Alumina (Merck) deactivated	4.3	366*	196	900	750	150	5/1
Bauxite	5.2	188	196	1200	975	225	4.3/1
Calcium carbonate	6.5	155	190	1023	831	192	4.1/1
Calcium phosphate	4.2	180	176	1230	1020	210	5/1
Calcium monohydrogen phosphate	4.4	168	162	1170	990	180	55/1
Calcium dihydrogen phosphate	3.7	152	170	1190	1005	186	5.3/1
Talc	7.8	147	142	1200	960	240	4/1
Sodium carbonate							
(i) Commercial heated to 105°C for 2 hr	12.6	248	253	1020	930	90	15/1
(ii) Anhydrous (Merck)	5.8	288	290	1080	960	120	8/1
Starch (Rafhan) S	5.9	300	306	1530	1080	450	2.4/1
Sugar	6.3	292	296	1680	1215	465	2.6/1

TABLE 2.—RECOVERY OF PURE β -CAROTENE BY COLUMN CHROMATOGRAPHY USING DIFFERENT ADSORBENTS. (ADSORBENTS (10 g) β -CAROTENE ADDED TO THE COLUMN. (0.1 mg)).

Adsorbents	Length of column (cm)	Amount of β -Carotene recovered (mg)	Percent recovery
Alumina	4.5	0.099	99.0
Bauxite	4.5	0.098	98.0
Starch	7.5	0.099	99.0
Sugar	7.0	0.010	100.0
Calcium phosphate	7.5	0.094	94.0
Calcium carbonate	8.5	0.076	76.0
Talc	1.5	0.070	70.0
Sodium carbonate (commercial)	7.5	0.099	99.0
Calcium monohydrogen phosphate	7.5	0.094	94.0
Calcium dihydrogen phosphate	7.5	0.095	95.0

TABLE 3.—CAROTENOID AND CHLOROPHYLL CONTENTS OF ALFALFA (BERSEEM) AS OBTAINED BY SHAKING THE EXTRACT WITH VARIOUS ADSORBENTS. (VOLUME OF EXTRACT USED. (10 ml)).

Adsorbent	Moisture %	Amount of adsorbent required (g)	Carotenoids mg/kg	Chlorophylls (mg/kg)
Alumina	4.3	8.5	195	890
Sugar	6.3	15	200	1650
Starch	5.9	14	302	1520
Sodium carbonate:				
(i) Commercial	12.5	8	270	1030
(ii) Anhydrous	6.0	7.5	275	1042

TABLE 4.—EFFECT OF REGENERATION BY DRYING AT 85°C (2 hr) AND WASHING WITH LIGHT PETROLEUM (60–80°) ON THE ADSORPTIVE CAPACITY OF VARIOUS ADSORBENTS.

Adsorbent	No. of times used	Regeneration by solvent washing				Regeneration by drying			
		Carotenoids mg/kg		Chlorophyll mg/kg		Carotenoid mg/kg		Chlorophylls mg/kg	
		Total	a	b	Total	a	b		
1. Sugar	1	292	1668	1215	453	293	1668	1119	477
	2	292	1683	1221	462	292	1680	1218	462
	3	294	1683	1221	462	292	1689	1224	465
	4	290	1689	1224	465	290	1698	1230	468
	5	297	1701	1227	474	295	1710	1230	480
	6	296	1710	1233	477	295	1710	1233	477
2. Starch	1	300	1512	1080	441	300	1524	1092	432
	2	301	1512	1080	432	300	1542	1098	444
	3	300	1533	1080	453	300	1551	1101	450
	4	307	1539	1086	453	305	1560	1101	459
	5	308	1551	1089	463	306	1560	1101	459
3. Sodium Carbonate (anhydrous)	1	228	1068	960	108	228	1062	906	102
	2	229	1080	960	120	226	1083	972	111
	3	232	1080	960	120	232	1086	972	114
	4	234	1095	969	126	236	1101	975	126
	5	236	1119	978	141	236	1110	981	129
4. Sodium Carbonate commercial	1	248	1017	921	96	248	1032	936	96
	2	245	1047	936	111	250	1041	936	105
	3	252	1059	945	114	250	1059	939	120
	4	252	1080	954	126	255	1071	948	123

The difference in chlorophyll contents is worth noting and it varies from 900 to 1680 mg/kg. The ratio of chlorophyll a to chlorophyll b also varies from 2.4 to 15. It is generally believed that this ratio in higher plants is about 2 which is obtained in case of starch and sugar. The increase in this ratio means less recovery of chlorophyll b which due to its higher polarity, is adsorbed more

strongly. It is mentioned here that elution of chlorophylls fraction with 30% acetone in light petroleum leaves the adsorbents like sugar and starch almost colourless which indicates that whole of chlorophylls are eluted. The other adsorbents retain a greenish tinge which is not washed even with higher contents of acetone in the eluting mixture.

Table 3 shows that the amounts of adsorbents vary according to their activity. The results obtained in this method closely resemble with those of column method. It clearly indicates that for large scale experiment, the simple method would be to shake the adsorbent with the extract in some container and separate the supernatant liquid.

For the economical production, the adsorbent should be used several times before repacking the column. Table 4 shows the number of times the adsorbent can be efficiently used. If the adsorbents are used beyond this limit, carotenoids and chlorophylls bands tend to come together. It is interesting to note that solvent washing and drying are equally efficient in the regeneration of the adsorbents. It will be noted that there is small increase in the amount of both carotenoids and chlorophylls eluted through the columns repeatedly being used for a number of times showing thereby the deactivation of the adsorbent after each use.

Acknowledgement.—The authors are grateful to Mr. Mohammad Arshad for his technical assistance in the purification and recovery of solvents. The authors are also thankful to Dr.

M. Haneef Qureshi, Dr. M. Saeed and Mr. Aziz-ul-Haque for supplying the samples of talc and bauxite and calcium phosphates respectively. Thanks are also due to Mr. M. Aslam, Director, PCSIR Laboratories, Lahore for his keen interest in the project.

References

1. M.A. Shah, A. Kabir Qureshi and Manzoor Elahi, Pakistan J. Sci. Ind. Res., (1969).
2. M.A. Shah, A. Kabir Qureshi and Manzoor Elahi, Science and Industry (under publication).
3. M.E. Wall, E.G. Kelly and J. J. William, Ind. Eng. Chem., **36**, 1057 (1944).
4. W.H. Shearon Jr. and O.F. Gee, Ind. Eng. Chem., **14**, 218 (1949).
5. T.B. Mann, Analyst., **69**, 34(1944).
6. F.P. Zscheile and C.L. Comar, Botan. Gaz., **102**, 463(1941).
7. H.L. Mitchell, W.G. Schrank and E.R. Silber, Ind. Eng. Chem., **45**, 415 (1953).
8. A.R. Kemmerer and G.S. Fraps, Ind. Eng. Chem., **15**, No. 12, 714 (1943).
9. F.P. Zscheile and C.L. Comar, Plant Physiol., **17**, 198 (1942).

In the second set of experiments hydrogen chloride was made by treating the sample with 10% hydrochloric acid. The contents was shaken for five

from aqueous solution. Maclean and Kertész reported that soil acidity and ion exchange properties of soils were due to clay content. The absorption of cations by a clay mineral does not involve any structural change in the mineral. In the process one cation is taken up from the solution and another is released in the solution. Exchange is a reversible process between two types of cations so the process is known as cation exchange process. Total amount of cation exchange taken place in 100 g of clay is known as cation exchange capacity (c.e.c.) of the clay. It is very important for a neutral clay to absorb several cations. Common ones are H⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺ and Al³⁺. It is customary to distinguish between clay in which one cation is predominantly or exclusively absorbed. Thus a sodium clay is one in which the absorbed cation is sodium and it may be represented as (Na) and the exchange reaction between two cations may be expressed as (Na)X + NH₄⁺OH = (NH₄)X + NaOH. C.e.c. is an important physical property and it has the relation with various important physical characteristics of the clay, e.g. plasticity, water uptake, drying property, viscosity of a clay slip, etc. particularly when the clay is in the water medium. The c.e.c. value provides useful information to the chemist as to the nature of the minerals in a clay.

With a view to get adequate knowledge of work-
ing properties of some local Pakistani clays, c.e.c.