Decoloration of Vegetable Oils and Oleoresins with Recovery of Unaltered Pigments

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The present study confirms that N,N-dimethylformamide for the extraction of chloroplast pigments from vegetable tissues shows no differences from the usual acetone or methanol. Therefore, it can be applied to fats, as it allows separation of lipids and pigments by means of phase distribution between light petroleum ether and N,N-dimethylformamide. The ether phase retains the decolored fatty matter, and the pigments dissolved in N,N-dimethylformamide can be recovered totally unaltered.

This method has been applied to oleoresins and oils from different products and origins. Satisfactory results have been obtained in terms of the degree of decoloration and the percentage of oil recovered. At the same time, the unaltered pigment concentrate obtained from the hypophase could be used as a color enhancer in the chemico-pharmaceutical industry.

KEY WORDS: Carotenoid (recovering), chlorophyll, N,N-dimethylformamide, oil, oleoresin (decoloration), pigments.

The complex composition of the olive fruit, *Olea europaea* (1), creates serious problems for the isolation, identification and quantitation of chlorophylls, because the characteristic fatty matter of the olives prevents the application of the usual techniques recommended in the literature (2–4).

With the double aim of evaluating chlorophylls and carotenoids in the olive fruit during the growth and development stages and to know the effects of the extraction process of virgin olive oil on those pigments, a technique was perfected to eliminate the lipid components that interfere with and slow up analyses (5,6). Once the pigments have been extracted with N,N-dimethylformamide, the system of phase distribution between N,N-dimethylformamide and hexane is an effective procedure for the purification of chlorophylls and xanthophylls, which remain solubilized and unaltered in the N,N-dimethylformamide phase without interference from the fatty matter, which is retained in the hexane phase.

The present study attempts to elucidate the possibilities of applying this methodology to fats and oils, allowing decoloration of oils with a high concentration of pigments, as well as recovery of the latter totally unaltered. The usefulness of the phase distribution technique is verified by means of its application to highly colored vegetable oils and aromatic oleoresins, introducing slight modifications in the original method perfected for the study of chlorophylls in olive fruit.

Separating these unaltered pigments would be of great interest for a possible commercial application because of the use of controversial antioxidants in food oils. In addition, the provitamin, anti-cancer and anti-ulcer properties recently attributed to the carotenoids make them very attractive to the chemico-pharmaceutical industry (7,8).

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MATERIALS AND METHODS

Materials. For evaluating the extraction power of N,N-dimethylformamide, leaves of the beet, *Beta vulgaris*, of the family Chenopodiaceae, were used. The distribution of fatty matter between hexane and N,N-dimethylformamide was determined with commercial corn oil, which is almost uncolored and thus avoids possible pigment interference. Finally, the efficiency of the method was confirmed with aromatic oleoresins supplied by Industrias Bordas y Chinchurreta, S.A. (Sevilla, Spain), and with pepper seed oil obtained from the experimental plant of the Instituto de la Grasa (Sevilla, Spain).

Pigment extraction. To evaluate the degree of extractive exhaustion permitted by N,N-dimethylformamide, compared with acetone and methanol, quadruplicate extractions of beet leaves were made with each of the solvents. The sample (2.000 g) was weighed and treated with 50 mL of solvent in a trituration homogenizer at 5000 rpm. The mixture was vacuum filtered and the solid residue was treated again with 50 mL of solvent. The extraction was repeated until completely colorless filtrates were obtained. The filtrates were united and the pigments were transferred to ethyl ether for concentrating under vacuum at a temperature below 30°C. The dried residue was redissolved in 5 mL of acetone.

Pigment and fatty matter separation. The method perfected by Minguez *et al.* (4) for virgin olive oil and adapted to oleoresins and highly colored seed oils is described below.

Approximately 0.500 g of sample was weighed and dissolved in 70 mL of N,N-dimethylformamide. It may be necessary to use an ultrasonic bath for a perfect solution. The solution was passed to a decanting funnel, and 50 mL of light petroleum ether was added. The mixture was stirred and left to settle until complete phase separation. Each of the phases was subjected to four further treatments in a countercurrent system in order to achieve maximum retention of fatty matter in the epiphase and pigments in the hypophase.

The five resulting fractions of N,N-dimethylformamide were combined and passed to a decanting funnel, in which 200 mL of aqueous Na_2SO_4 (2%) at approximately 0°C, and 100 mL of ethyl ether, had been placed previously. The mixture was stirred vigorously and left to settle until complete phase separation. The pigments were transferred to ethyl ether. The uncolored aqueous phase was discarded and the ether phase was evaporated to dryness at a temperature below 30°C at reduced pressure. The residue was collected in 10 mL cyclohexane, and kept in a topaz-colored container in a refrigerator for later study.

The light petroleum ether phases were similarly combined in a decanting funnel and washed with aqueous Na_2SO_4 (2%). The aqueous phase was discarded and the

organic phase was evaporated to dryness at a temperature below 30°C at reduced pressure. The weight of dry residue of this phase was used as a control on the percentage of fatty matter retained. The pigments included in this phase may be studied later by double-development by thin-layer chromatography (TLC) or saponification of the extract to eliminate the lipids.

This last operation was carried out as follows: The fatty residue was dissolved in 100 mL ethyl ether and treated in a decanting funnel with 100 mL of a 20% solution of KOH in methanol. Distilled water was added to break the phases after one hour, with the pigments passing to the ether phase and the soaps formed to the aqueous phase. The ether phase was washed to neutrality, and dried with an aqueous solution of sodium sulfate (2%). This was concentrated to dryness under vacuum and a temperature below 30°C. The final residue was dissolved in a small volume of acetone or cyclohexane and kept in darkness in a refrigerator unless it was used immediately.

Pigment separation. It was carried out by thin-layer chromatography (TLC) on silica gel plates 60 GF_{254} (0.7 mm thickness), with the following developers: petroleum ether (65–95°C)/acetone/diethylamine (10:4:1); hexane/ethyl acetate/acetone/ethanol (95:3:2:2); petroleum ether (65–95°C)/acetone/pyridine (10:4:2.5); hexane.

Pigment identification. The absorption spectra, as well as adsorption properties and the color shown by the substances in TLC under white and UV light, served as a basis for identification of chlorophylls and derivatives (2). Standards of chlorophyll "a" and "b" were Sigma numbers C-6144 and C-3878. Standards of pheophytins "a" and "b" were prepared in the laboratory according to the procedure described by Jones *et al.* (9), and pheophorbide standards were made according to Hynninen (10). For carotenoids, the adsorption properties in TLC (before and after saponification) and the absorption spectra in the visible and absorption bands in the infrared (IR) were taken into account. For confirmation of functional groups, distinct physicochemical reactions specified in the literature were assayed (11). Standards of β-carotene and lutein were obtained from Lucerne (12), violaxanthin and neoxanthin from olives (5), and capsanthin, capsorubin, cryptoxanthin and zeaxanthin from red pepper (13).

Quantitation. Once the chromatographic development of a known quantity of pigment extract was finished, each substance was scraped from the plate, eluted with acetone, and made up to a determinate volume. Next, the respective absorption spectrum was obtained, and the extinction value E_o, at the maximum absorption wavelength, was substituted in the equation $E = E_o \cdot C$. The resulting concentration values were obtained as milligrams per kilogram of sample. In Table 1 (refs. 11,14,15) are shown the extinction coefficients of each pigment at the wavelength of maximum absorption.

TABLE 1

Absorption Maxima and Extinction Coefficients of Pigments in Solution

Pigment	λ_{max} (nm)	1% E_o 1 cm	Solvent	Ref.
β-Carotene	450	2592	Hexane	14
	450	2620	Acetone	15
Lutein	445	2540	Ethanol	11
	446	2340	Acetone	15
Violaxanthin	454	2216	Benzene	14
	440	2340	Acetone	15
Neoxanthin	438	2270	Ethanol	14
	438	2050	Acetone	15
Cryptoxanthin	445	2625	Hexane	14
Zeaxanthin	450	2340	Hexane	14
Capsanthin	484	1970	Benzene	14
Capsorubin	474	2165	Light petroleum ether	14
Chlorophyll "a"	662	670	Acetone	15
Chlorophyll "b"	646	518	Acetone	15
Pheophytin "a"	666	540	Acetone	15
Pheophytin "b"	654	350	Acetone	15
Pheophorbide "a"	668	510	Acetone/pyridine (1:1)	15
Pheophorbide "b"	660	400	Acetone/pyridine (1:1)	15

RESULTS AND DISCUSSION

N,N-dimethylformamide as extractant solvent of chloroplastic pigments. As N,N-dimethylformamide is an infrequently used solvent for extraction of chloroplastic pigments, a comparative study has been made of its extracting power against the usual ones—acetone and methanol. Green leaves of beets were used for the experiments. The results of the assay used for identification of the main pigments and the spectral properties in different solvents are detailed in Table 2. The individual contribution of each pigment in the extracts obtained from the different solvents is detailed in Table 3, as averages and confidence intervals of four replicates. In the same Table are shown the values of total chlorophylls and carotenoids, and the ratios of chlorophylls/carotenoids and chlorophyll "a"/chlorophyll "b". Those ratios should remain constant for any single product, whatever the solvent used, if the solvent is not selective.

The analysis of variance of the results for each individual pigment and fraction, as well as for the pigment ratios in the three studied solvents, shows that there are no significant ($p < 0.05$) differences among them. In other words, the three solvents show similar behavior. The presence of pheophytins is sporadic and due to sample manipulation. Their amounts are relatively low, less than 3.5% of the total chlorophyllic pigments. There are no significant statistical differences among the values found for the three solvents, but there is a clear tendency for them to be greater in acetone and methanol than in N,N-dimethylformamide due to the latter's high pH.

With N,N-dimethylformamide a total of three treatments is necessary to reach the endpoint, while in the case of acetone a much larger number of extractions is necessary. These studies affirm that these three solvents are similar, and that none have special selectivity.

Adaptation of the method. Once shown that N,N-dimethylformamide is a good extractor of chlorophyllic and carotenoid pigments, and that phase distribution achieves a good separation between lipids and pigments without pigment loss or destruction, the idea that this methodology could be converted into an alternative technique for the decoloration of oils began to take shape.

Control of fatty matter distribution carried out with corn oils shows a fat recovery in the hexane phase greater than 90%. With respect to pigment behavior, earlier studies carried out on virgin olive oil showed that chlorophylls and their derivatives plus xanthophylls were

TABLE 2

Characteristics Used for the Identification of the Main Pigments in Green Beet Leaves, Separated on Silica Gel with Petroleum Ether (65–95°C)/Acetone/Diethylamine (10:4:1)

Pigment identified		Spectral data λ_{max} (nm)			IR band		Epoxide test (HCl treatment)	
Carotenoids	RF value	Light petroleum ether	Peak ratio	Chloroform	−OH	Ester C=O	Hypsochromic shiff (ethanol) nm	Color on TLC
β-Carotene	1.00	(426),444,470	8%	(434),458,482	−	−	0	Yellow
Lutein	0.41	418,442,470	57%	430,452,482	+	−	0	Brown with a green border
Violaxanthin	0.28	414,436,466	73%	422,446,476	+	−	40	Blue
Neoxanthin	0.15	412,436,466	60%	420,444,474	+	−	14	Green-blue

		Spectral data λ_{max} (nm)			Color in TLC	
Chlorophylls	Rf value	Ethyl ether	Acetone	Peak ratio	White light	UV
Chlorophyll "a"	0.51	430,615,662	428,616,662	1.4	Blue green	Pink fluorescence
Chlorophyll "b"	0.44	455,595,644	454,596,646	3.0	Yellow green	Pink fluorescence
Pheophythin "a"	0.57	408,471,667	410,468,668	2.4	Grey	Pink fluorescence

TABLE 3

Comparative Study of the Pigment Extraction Capacity of N,N-Dimethylformamide, Methanol and Acetone from Green Beet Leaves. (Average of Four Samples)

	Pigment concentration (mg/kg)		
Pigment	Acetone	N,N-dimethylformamide	Methanol
β-Carotene	50.08 ± 5.40	47.50 ± 11.93	50.72 ± 3.38
Pheophythin "a"	20.06 ± 4.12	13.60 ± 9.79	36.04 ± 34.75
Chlorophyll "a"	791.39 ± 74.46	844.06 ± 151.72	803.60 ± 97.86
Chlorophyll "b"	181.40 ± 47.49	177.78 ± 21.58	179.55 ± 17.95
Lutein	85.06 ± 3.27	84.36 ± 10.37	92.97 ± 16.32
Viloxanthin	51.11 ± 7.01	57.24 ± 6.05	48.82 ± 6.69
Neoxanthin	33.98 ± 7.04	33.74 ± 6.30	30.95 ± 5.17
Total chlorophylls	992.84 ± 107.79	1035.45 ± 180.34	1019.19 ± 148.98
Total carotenoids	220.23 ± 10.59	227.85 ± 28.51	223.46 ± 23.74
Ratio[a]	4.57 ± 0.99	4.81 ± 0.31	4.67 ± 0.36
Ratio[b]	4.50 ± 0.36	4.53 ± 0.23	4.55 ± 0.20

[a]Of chlorophyll "a"/chlorophyll "b".

[b]Of chlorophylls/carotenoids.

solubilized in N,N-dimethylformamide, and that the pigmentation retained in the hexane phase was due almost exclusively to β-carotene (16).

When this method was applied to pepper seed oil, highly colored and with a pigment composition completely different from that of the olive fruit, it was found that there was a different distribution of fatty matter and pigments between the solvents mentioned. The fat remains retained in the hexane, which is colored red. TLC with double development [the first with hexane to remove lipids, which advance to the front of the chromatogram together with β-carotene, and the second with the mixture hexane/ethyl acetate/acetone/ethanol (95:3:2:2)] shows that cryptoxanthin, zeaxanthin, capsanthin and capsorubin are also present. After testing different mixtures with hexane and light petroleum ether with the aim of modifying the epiphase polarity, TLC of the same oil showed that on using light petroleum ether in place of hexane, capsanthin was found only in traces, and capsorubin had disappeared, while cryptoxanthin and zeaxanthin remained.

Consequently, the behavior of corn oil with the mixture of light petroleum ether and N,N-dimethylformamide was studied with the aim of controlling the retention of the fatty matter. The results were similar to those with hexane. The scheme of the proposed method for separation of lipids and pigments is shown in Figure 1.

Application of the method. Having shown that light petroleum ether retained most of the fat and little of the pigments, this technique was applied to pepper seed oil and various oleoresins. The identified pigments and their distribution in the phase of light petroleum ether and N,N-dimethylformamide are shown in Table 4.

Chlorophyll and carotenoid fractions were quantitated by using the extinction coefficient of the major pigment

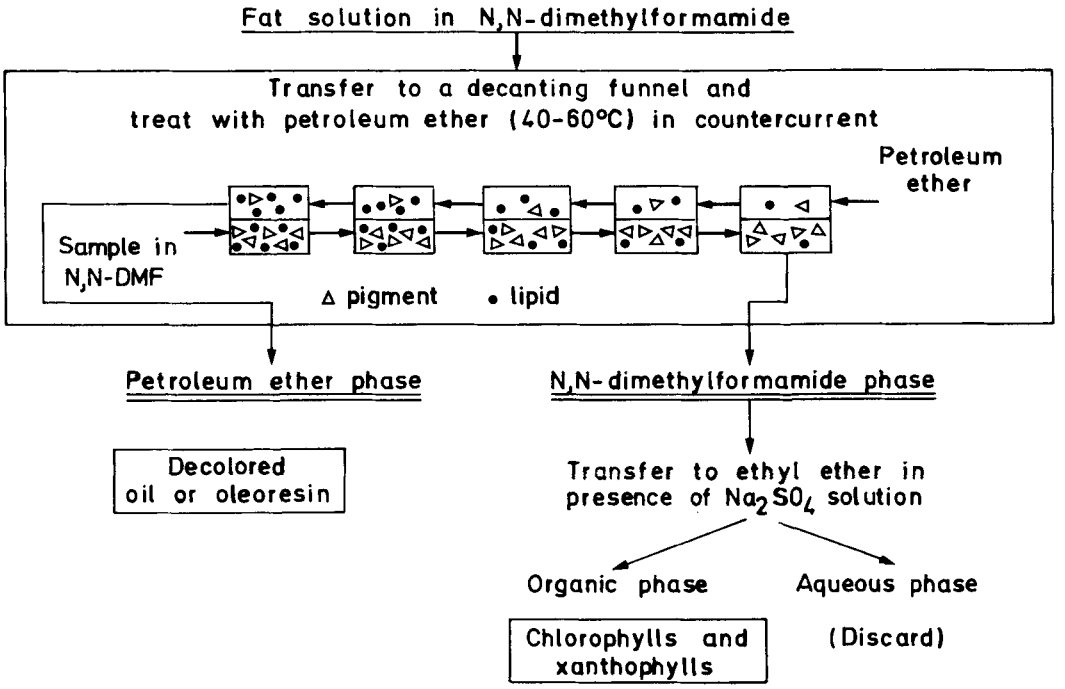

FIG. 1. Proposed method for separation of lipids and pigments.

TABLE 4

Pigments from Vegetable Oils and Oleoresins Identified in the Light Petroleum Ether (LPE) and N,N-Dimethylformamide (N,N-DMF) Phases, Separated by TLC on Silica Gel 60 GF_{254}

Pigment identified	Rf value	Spectral data λ_{max} (nm) LPE	Peak ratio	Pepper seed oil		Aromatic oleoresins	
				LPE	N,N-DMF	LPE	N,N-DMF
Carotenoids							
β-Carotene	1.00[a]	(426),444,470	8.0%	+++		+++	
Cryptoxanthin	0.86[b]	(422),446,472	15.0%	++	+		
Zeaxanthin	0.76[b]	(422),446,474	22.0%	++	+		
Capsanthin	0.37[b]	468,496	6.0%	+	++		
Capsorubin	0.21[b]	444,468,502	29.0%		+++		
Lutein	0.23[a]	418,442,470	57.0%				+++
Chlorophyllic derivatives		Acetone					
Pheophytin "a"	0.45[a]	410,468,668	2.4%				+++
Pheophytin "b"	0.33[a]	432,522,656	5.1%				+++
Pheophorbide "a"	0.45[c]	410,468,668	3.1%				+++
Pheophorbide "b"	0.34[c]	432,522,656	8.0%				+++
Pyropheophytin "a"	0.48[a]	410,468,668	2.4%			+	++
Pyropheophytin "b"	0.40[a]	432,522,656	5.5%			+	++

Solvent system: [a]Petroleum ether (65–95°C)/acetone/diethylamine (10:4:1). [b]Hexane/ethyl acetate/ethanol/acetone (95:3:2:2). [c]Petroleum ether (65–95°C)/acetone/pyridine (10:4:2.5).

+++Total.

++Partial.

+Traces.

TABLE 5

Distribution of Fatty Matter and Chlorophyllic and Carotenoid Pigments Between Light Petroleum Ether (LPE) and N,N-Dimethylformamide Phases (N,N-DMF)

Sample	Fatty matter (%) Phase		Carotenoid pigments (%) Phase		Chlorophyll pigments (%) Phase	
Seed oils from	LPE	N,N-DMF	LPE	N,N-DMF	LPE	N,N-DMF
Zea mays L.	94.17	5.83	—	—	—	—
Capsicum annuum L.	81.70	18.30	16.00	75.00	—	—
Oleoresins from						
Mentha arvensis L.	63.14	36.86	5.70	90.00	1.30	94.70
Origanum vulgare L.	64.50	35.50	0.10	66.60	2.85	75.35
Thymus mastichina L.	60.91	39.09	7.60	52.27	3.40	68.38
Salvia officinalis L.	60.65	39.35	5.89	66.50	0.30	68.50
Thymus vulgaris L.	68.40	31.59	5.70	74.91	2.30	86.67

in order to be able to complete the quantitation directly in the total absorption spectrum. The measurement was taken at the characteristic wavelength of pheophytin "a" and β-carotene in the light petroleum ether phase and pheophorbide "a" and lutein in the N,N-dimethylformamide for oleoresins. In the case of pepper seed oil the major pigment in the top phase is β-carotene and capsanthin is prominent in the bottom one. For greater simplicity, the results are expressed in Table 5 as percentages of pigments retained in each phase with respect to the content in the initial sample.

The proportion of fatty matter retained in the light petroleum ether phase ranges from a high of 81% to 60% in the least favorable case. For oils with single lipid composition, results close to 100% were obtained, as with corn oil. When using processed samples of greater complexity, such as oleoresins, retention of fatty matter is somewhat greater, due probably to its natural heterogeneity or the formation of highly polar compounds by thermal oxidation of fatty acids during the production of the oleoresin.

With respect to pigment distribution, the sum of percentages did not reach 100% in any case. Therefore, we investigated if some coloration was eliminated up on applying the phase distribution process. In fact, the existence of water-soluble colorants has been shown, probably of a polyphenolic nature.

Color retention in the ether phase is relatively low. In all cases, β-carotene is the major carotenoid pigment. The chlorophyllic pigments detected in this phase are always less than 5% of the total, and their low polarities point to highly degraded chlorophyllic derivatives. Thus, the color of this phase is directly attributable to the β-carotene content of the initial product, and in lesser measure to degradation products of the chlorophylls.

The greater part of the pigments, both chlorophylls and carotenoids, are solubilized in N,N-dimethylformamide. In the aromatic oleoresins, the chlorophyllic fraction is mainly composed of pheophytins and pheophorbides "a" and "b", while the carotenoid fraction is composed almost exclusively of lutein.

Pepper seed oil has a different composition than the other products studied, so that besides β-carotene, the carotenoids cryptoxanthin, zeaxanthin and, in lesser measure, capsanthin, are retained in the petroleum ether, because when esterified they show greater affinity for this phase. In the N,N-dimethylformamide phase for pepper seed oil, capsanthin and capsorubin are retained as principal pigments—these being carotenoids found exclusively in pepper. The rest of the highly polar carotenoids are also found, but they have not been given much attention because they cause no problems in the application of the methodology. β-Carotene was never present in this phase in any of the extractions.

The results obtained showed that this methodology could be used as an alternative for the decoloration of oils, allowing for the recovery of the pigment at the same time, in a totally unaltered state. These pigment products may be useful in the chemico-pharmaceutical industries.

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REFERENCES

1. Vázquez-Roncero, A., *Grasas. y Aceites 14*:262 (1963).
2. Smith, J.H.C., and A. Benítez, in *Modern Methods of Plant Analysis*, Vol. 4, edited by K. Paech and M.V. Tracey, Springer-Verlag, Berlin, 1955.
3. *Official Methods of Analysis of the AOAC*, 14th edn., Association of Official Analytical Chemists, Arlington, Virginia, 1984.
4. *Official and Tentative Methods of the American Oil Chemists' Society*, 3rd edn., American Oil Chemists' Society, Champaign, IL 1988, Method Cc. 13d-55.
5. Mínguez-Mosquera, M.I., and J. Garrido-Fernández, *J. Agric. Food Chem. 37*:1 (1989).
6. Mínguez-Mosquera, M.I., B. Gandul-Rojas and J. Garrido-Fernández, *J. Am. Oil Chem. Soc. 67*:192 (1990).
7. Carlson, B.L., and M.H. Tabacchi, *J. Food Sci. 51*:218 (1986).
8. Fakourelis, N., E.C. Lee and D.B. Min, *Ibid. 52*:234 (1986).
9. Jones, I.D., L.S. Butler, E. Gibbs and R.C. White, *J. Chromatogr. 70*:87 (1968).
10. Hynninen, M., *Acta Chem. Scand. 27*:1771 (1973).
11. Davies, B.H., in *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, edited by T.W. Goodwin, Academic Press, London, New York, San Francisco, 1976.
12. Goodwin, T.W., in *Chemistry and Biochemistry of Plant Pigments*, Vol. 1, edited by T.W. Goodwin, Academic Press, London, New York, San Francisco, 1976.
13. Mínguez-Mosquera, M.I., and J. Garrido-Fernández, *Grasas y Aceites 35*:35 (1984).
14. Foppen, F.H., *Chromatogr. Rev. 14*:133 (1971).
15. Mínguez-Mosquera, M.I., J. Garrido-Fernández and B. Gandul-Rojas, *J. Agric. Food Chem. 37*:8 (1989).
16. Mínguez-Mosquera, M.I., and J. Garrido-Fernández, *Grasas y Aceites 36*:376 (1985).

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