Effect of nitrogen, phosphorus and flower stage of development on carotenoids and oil content of marigold (*Tagetes patula* L.)

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ABSTRACT

Seeds of marigold collected from local ornamental plants were grown at the farm of the Faculty of Agricultural Sciences, University of Gezira, Wad Medani, Sudan, during the 4th of March, 2007. The objective of the study was to investigate the effect of N, P and flower stage of development on carotenoids and oil contents of marigold (*Tagetes patula* L.). The study consisted of three rates of N (0, 43 and 86 kgha^{-1}) and two P levels (0, 41 kg P_2O_5 ha^{-1}). Flowers were harvested at the unopened, newly opened, mature and over mature stages. Treatments were arranged in a randomized complete block design with three replicates. Nitrogen and phosphorus fertilizers significantly increased plant height and number of branches of marigold. However, they did not affect flower carotenoids content. Marigold flowers contained about 5 different carotenoids including lutein, the major component and β-carotene. When compared with salad rocket which is a lutein rich plant, most of the lutein of marigold flowers was present in the esterified form in contrast to rocket leaves, in which case lutein was largely in the free form. Five carotenoids types appeared in marigold flowers 15 days after flower opening and less types were observed in younger and older flowers. The seeds of marigold contained 1.5% of fixed oil, the major fatty acid of which was linoleic acid.

INTRODUCTION

Many color additives used in foods and drugs are of synthetic origin. Synthetics, as compared to natural products, are cheaper to manufacture. However, this economic advantage is belittled by the potential health hazards inherent in the chemical structures of synthetics as their manufacturing methods do not usually preclude the formation or
removal of the unwanted optical isomers .In view of this, synthetic colorants are being continually removed from the market due to health hazards discovered after their approval of use (Munger,1988).

The major source of color additives is plants, which constitute a renewable resource. Food and drug colorants of plant origin include chlorophylls, anthocyanins and carotenoids. The latter play additional role, as some of them such as beta-carotene is the precursor of vitamin A (Bailey and Chen, 1989).

Plant carotenoids have been commercially used as an additive to laying hens feeds to colour their egg yolk (Tyczkowski and Hamilton, 1986; Couch and Farr, 1971).Marigold is grown as an ornamental plant and its flower petals are a good source of carotenoids pigments, especially xanthophylls (Seddon, 1994).

In Sudan, marigold carotenoids have been used by incorporating the powdered dried petals in the feed of chicken. Till now, there is no research on horticultural production aspects of marigold flowers in Sudan. Therefore, the objectives of this work were to study the effect of nitrogen and phosphorus fertilizers on growth of marigold and the optimum harvest time of the flowers.

MATERIALS AND METHODS

Nitrogen and phosphorus fertilizer experiment

Field experiments were carried out at the nursery of the Department of Horticultural Sciences, Faculty of Agricultural Sciences, University of Gezira, Wad Medani, Sudan. The land was ploughed, leveled and made into 70 cm ridges. The plot size was 2 x 2.3 m. Seeds were sown on the 4th of March, 2007. Seeds were sown 5 cm deep in the middle of the ridge at an inter-row spacing of 15 cm. Irrigation was applied 7-10 days according to weather conditions. Nitrogen in the form of urea was applied at the rates of 0, 43 and 86 kgNha^{-1}. Phosphorus was applied at 0 and 41 kg P_2O_5 ha^{-1}.The experiment was arranged in a randomized complete block design with three replicates.

Flowers were harvested at different stages; unopened flowers (*ca*.1-2 days old), flowers that had just opened (3-4 days), fully mature ones (*ca.*15 days) and over-mature flowers (>15 days old) and were taken forpigment analysis. Data collected consisted of plant height and number of branches per plant.

Extraction of carotenoids

Two methods were used for the extraction of carotenoid pigments from dried and powdered marigold flowers, with or without saponification of esterified xanthophyll carotenoids (Harbone, 1973), as follows:

a) A sample of 0.5 g of powdered marigold flowers, salad rocket and carrot root were allowed to stand in 5 ml of chloroform/ methanol (2:1) overnight at room temperature. The extract was filtered and concentrated before proceeding to further analysis.
b) Carotenoids extracted as above were taken to complete dryness, 1 ml of ethanol saturated with KOH was added and left to stand in the dark overnight at room temperature (or in a waterbath at 60°C for different intervals of time). Free carotenoids were extracted with hexane. The solvent was evaporated before subsequent analysis.

Thin layer chromatography: Preparation of plates

Thin layer chromatography (TLC) layers used were 0.25 and 0.5 mm thick. They were prepared using equipment from Shandon Scientific Instruments Ltd. A weighed amount of silica gel (G and 60 GF 254, MERCK KGaA, Germany) containing 13% $CaSO_4$ was shaken vigorously for about one minute with a volume of distilled water equivalent to twice the weight of the gel and applied to 20 × 20 cm glass-plates set at the required thickness. The plates were heated in an oven for 30 min at 110°C before cooling in a desiccator. The samples were applied to the plates and equilibrated against the developing solvent before chromatographic development.

A few micro liters of the prepared extract(s) were used in a solution for TLC separation. The solution was applied as a band, using a micro syringe, on a TLC plate coated with silica gel (0.5 mm thick). The plate was developed in a tank containing the solvent mixture dichloromethane: ethyl acetate (4:1) or hexane: acetone (85:15) for about 45 min. After solvent drying at room temperature, the developed plate was covered with another clean glass plate exposing a silica gel zone of about 2 cm at one edge. The two plates were clamped together and provisions were made to ensure that only narrow zones have been reached by the detection reagen anisaldehyde. Different separated bands were detected in the exposed edge using the reagent. The corresponding bands which were underneath the covered plate were scraped individually with care, transferred to a conical flask (100 ml) and eluted with chloroform : methanol (2:1) mixture. After filtration, the solvent was evaporated and kept for further analysis using an infra-red Spectrophotometer.

Lipid analysis: Extraction of seed fixed oil

Determination of oil content was carried out according to AOCS (1993). Marigold seed samples used for the determination of oil content were allowed to dry at room temperature. The seeds were thoroughly cleaned from flowers attachments before oil extraction, then crushed to fine particles using a mortar and pestle to ease the extraction process which continued for six hours in a Soxhlet extractor using n-hexane (-^oC – ^oC) b.pt. The solvent was recovered from the oil by a rotary evaporator and the oil percentage was calculated using the following equation:

$$\text{Oil}\,(\%) = \frac{\text{weight of oil}}{\text{weight of sample}} \text{ x } 100$$

Physico-chemical characteristics of marigold fixed oil

Marigold oil physical-chemical characteristics, namely, acid value, saponification value, peroxide value and unsaturation index were determined according to AOCS method (AOCS, 1993).

Transmethylation

Fatty acid methyl esters were derived using 0.5 N sodium methoxide, which was prepared by dissolving 11.5 g of sodium in one liter of methanol. About 0.5 g of the oil was weighed in a stoppered flask and 0.5 N sodium methoxide was added (7-8ml). The contents were heated for 3-5 mins in a water bath at 80^0 C with shaking and were transferred to a separatory funnel. A few drops of glacial acetic acid were added, followed by 15 ml of distilled water and 10 ml of n-hexane. After shaking, the n-hexane layers were collected and kept dry by the addition of anhydrous sodium sulphate. Before GLC analysis, the solvent was removed under pressure in a rotatory evaporator. Fatty acid standards were methylated similarly.

Gas-liquid chromatography (GLC): Oil fatty acid composition

The fatty acid composition of marigold seed oil, was determined by gas liquid chromatography (GLC) on prepared columns of polyethylene glycol adipate (PEGA) packing material silar (IOC = OV 275). (Goodwin, 1976) .The injector oven was held at 28°C, the detector at 250^0 C and the carrier gas (nitrogen) flow rate was 50 ml/min. The column oven temperature was maintained at 165^0 C. Peak areas were measured manually by triangulation. Marigold oil fatty acids were identified by comparing the retention times of their methyl esters with those of standard fatty acid methyl esters.

RESULTS AND DISCUSSION

Effect of N and P on growth parameters of marigold

Fig. 1 shows the effect of N and P on the height of marigold plant. Application of N at the rate of 86 kgNha^{-1} resulted in the tallest plants, followed by 43 kgN ha^{-1} and P application as compared to the control. These results are in agreement with previous findings of Singh and Rao (2005) who reported that nitrogen and phosphorus fertilizers increased the number of branches and number of flowers of marigold.

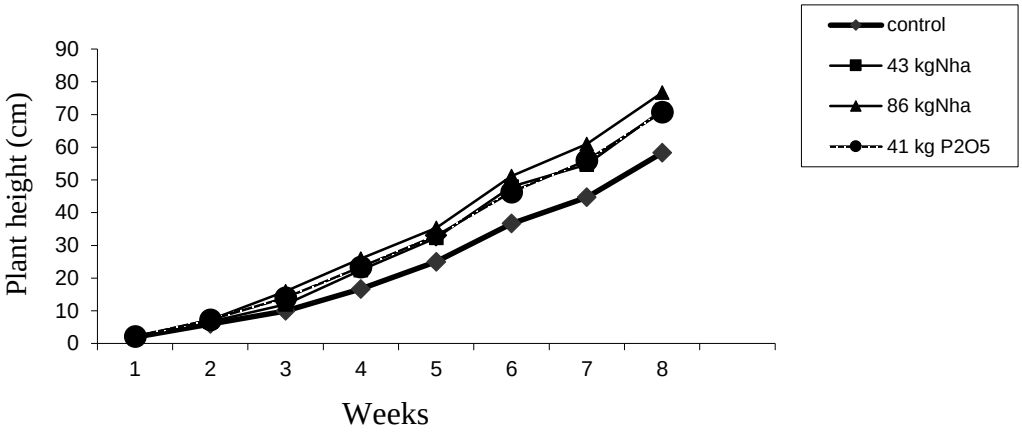


Fig.1.Effect of N and P fertilizers on the plant height of marigold.

Fig. 2 shows the effects of N and P on the number of branches of marigold. The higher rate of N resulted in the largest number of branches as compared to other treatments. Application of N at the lower rate resulted in the same number of branches as that obtained by the application of P alone.

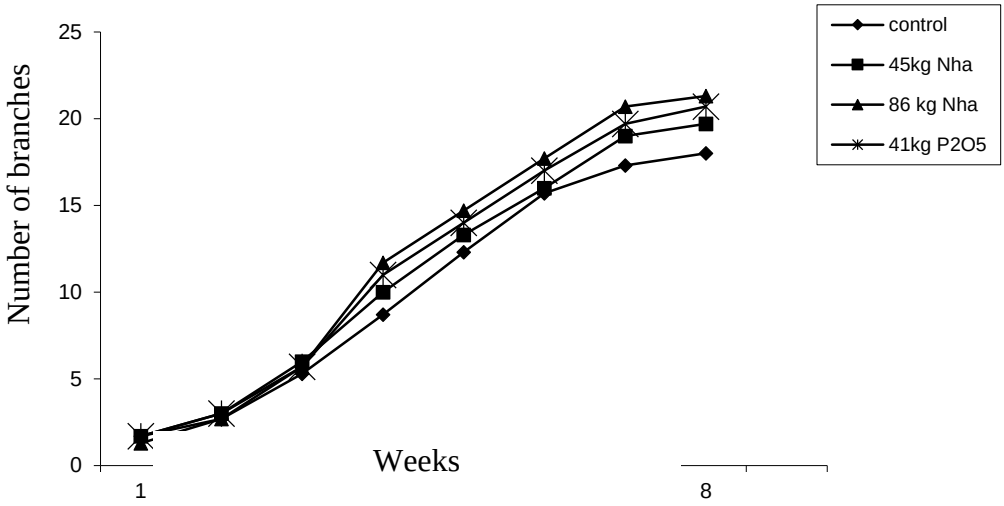


Fig.2. Effect of N and P fertilizers on number of branches of marigold.

Identification of carotenoid components

Fig. 3 shows that two free hydroxyl forms of carotenoids could be detected (spots 1 and 2). It is noteworthy that free (unestrified) carotenoids were present in very small amounts in marigold flowers (Fig. 3). It is also evident that small amounts of xanthophylls monoester were present in marigold flowers. The fact that most of the xanthophylls of marigold flowers are esterified has been reported by Harikumar *et al.* (2008).

The major carotenoid of marigold is lutein (Gomez and Macarulla, 1978). Other carotenoids present in small amounts were zeaxanthin, epoxylutein, flavoxanthin and chrysantheaxathin (Harbone, 1973). It is assumed, at this point, that spot 3 in Fig. 3 is likely lutein. It is known that salad rocket (*Eruca sativa* L). is quite rich in lutein and that carrot root is
rich in beta-carotene. Carotenoids pigments of both rocket leaves and carrot root were extracted and separated by TLC along with unsaponified and partially saponified marigold carotenoids extracts. Fig.4 shows that the major carotenoids of rocket leaves is likely lutein. It had the same Rf (50%) value as spot 2 of marigold extracts (Fig. 3). In carrot, the carotenoids running at solvent front (Fig. 4) would be beta-carotene, which is also present in marigold and rocket leaves. It is remarkable that xanthophylls of rocket were present largely in the free (unesterified) form, in contrast to those of marigold flowers which are esterified. No reports are available on esterified and nonesterified xanthophylls of rocket. Spot 4 of the partially saponified marigold extract is likely xanthophylls monoester, while spot 5 is the diester form.

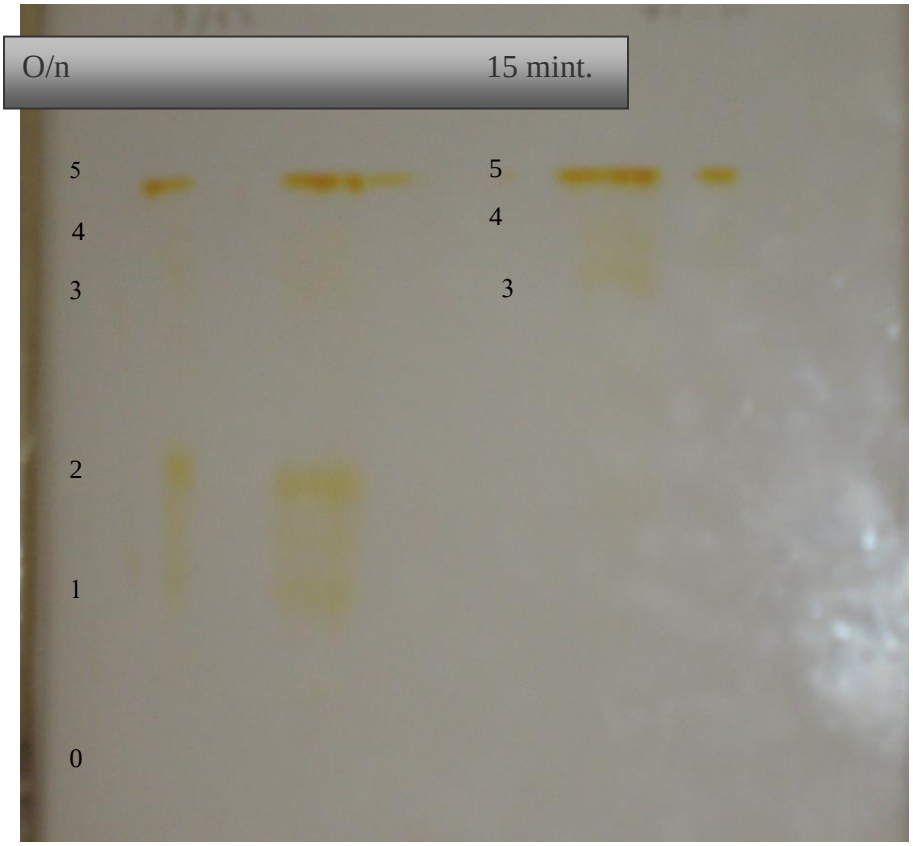


Fig.3. TLC separation of KOH hydrolyzed marigold extracts.

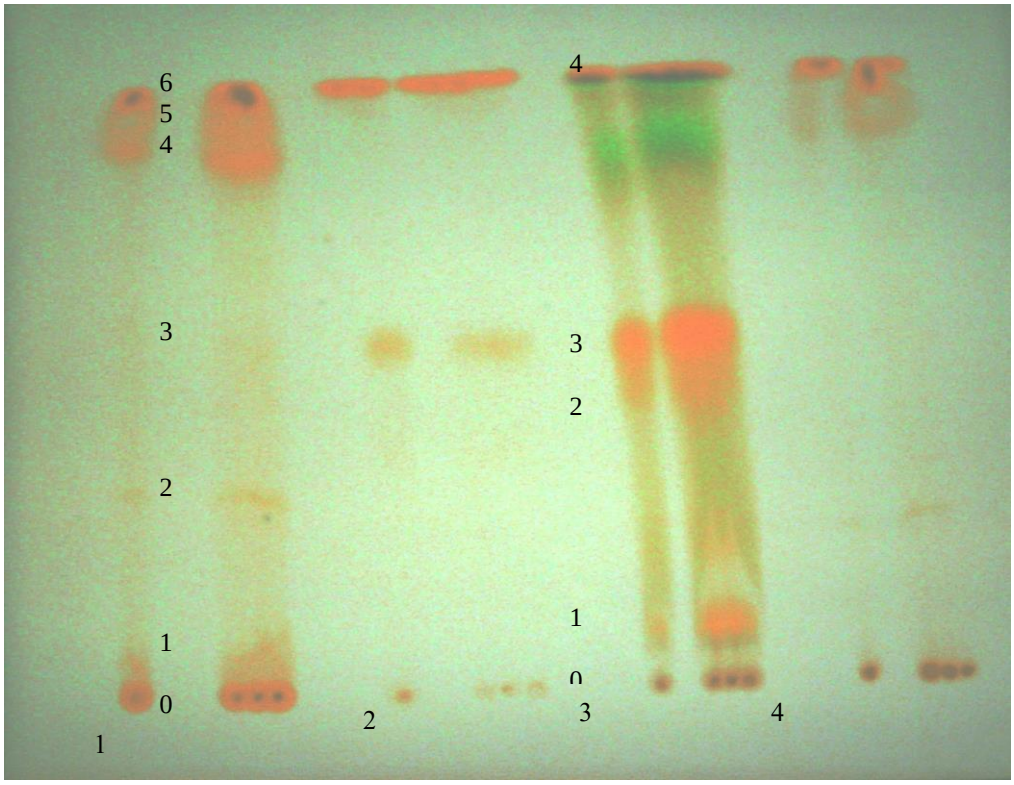


Fig. 4. TLC separation of partially saponified carotenoids of marigold (lane 1) and unsaponified carotenoids of carrot (lane 2), rocket (lane 3) and marigold (lane 4).

Tables 1, 2 and 3 show the infra red spectroscopic absorption of three compounds separated by TLC. Similar absorption spectra were observed for the three compounds. The three compounds showed I.R. absorbing groups present in xanthophylls (Dudley and Lan, 1980), e.g. (-OH), (C-$(CH3)_2$, (ester C-O) and double bonds.

Effect of N and P on carotenoids of marigold flowers

Fig. 5 shows qualitative TLC separation of marigold flower carotenoids as affected by N and P fertilizers. Application of N and P fertilizers had no significant effects on the quality of carotenoids of marigold flowers.

Table 1. Infra red absorption spectrum of compound 1 isolated from marigold flowers.

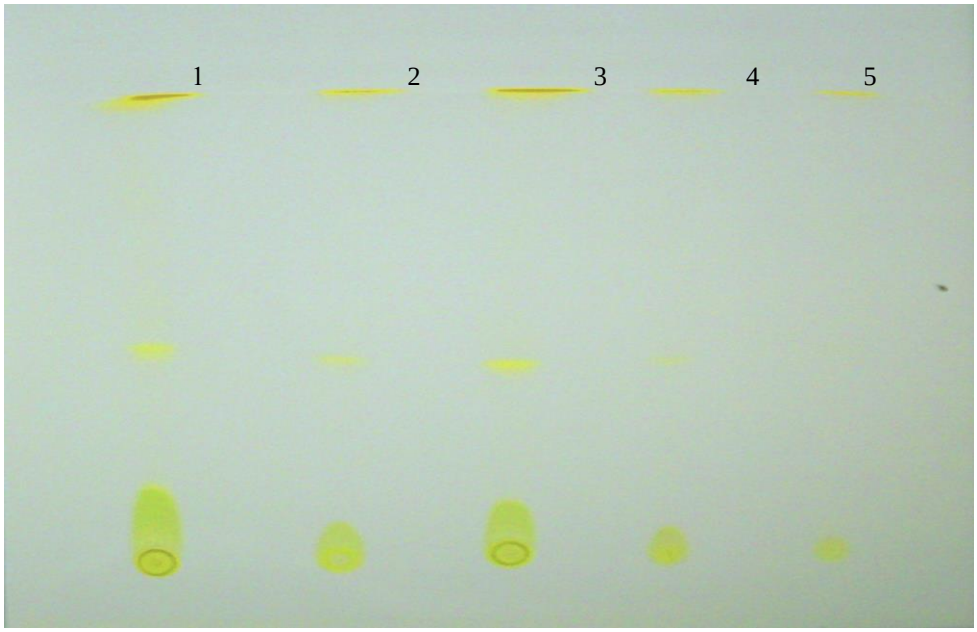
Frequency observed cm^{-1}	Possible identification of expected chemical group
3377	H – bonded [– O – H]
	OH – alcohol (generally)
2974	CH_2,-CH_3{C-H stretching}
2891	
1650	Enol ester or C = C
1450	CH_2,- CH_2{-C-H deformation}
1380	CH_3 or {C-$(CH_3)_2$}
1326	O – H [O – H bending]
1087	C-OH{C-O stretching}
1047	
758	Olefinic, C – H

Table 2. Infra red absorption spectrum of compound 2 isolated from marigold.

Frequency observed cm^{-1}	Possible identification of expected chemical group
3377	H – bonded [– O – H]
	OH – alcohol (generally)
2974	C-H_2,-CH_3{C-H stretching }
2891	
1647	C – C
1448	CH_2,-CH3{-C-H deformation}
1380	CH_3 or C-$(CH_3)_2$
1223	C-OH [C – O stretching]
1088	C-OH [O – H bending]
1047	
879	Olefinic, C – H
760	

Table 3.Infra red absorption spectrum of compound 3 isolated from marigold.

Frequency observed cm^{-1}	Possible identification of expected chemical group
3371	O – H [H – bonded]
	OH – alcohol (generally)
2974	CH_2,-CH_3{C-H stretching }
2891	
1647	C – C
1448	CH_2,CH_2{-C-H deformation}
1380	CH_3 or {C-$(CH_3)_2$}
1326	O – H [O – H bending]
1223	C-OH { C – O stretching}
1087	C-OH { O – H bending}
1047	
879	Olefinic, C – H
759	

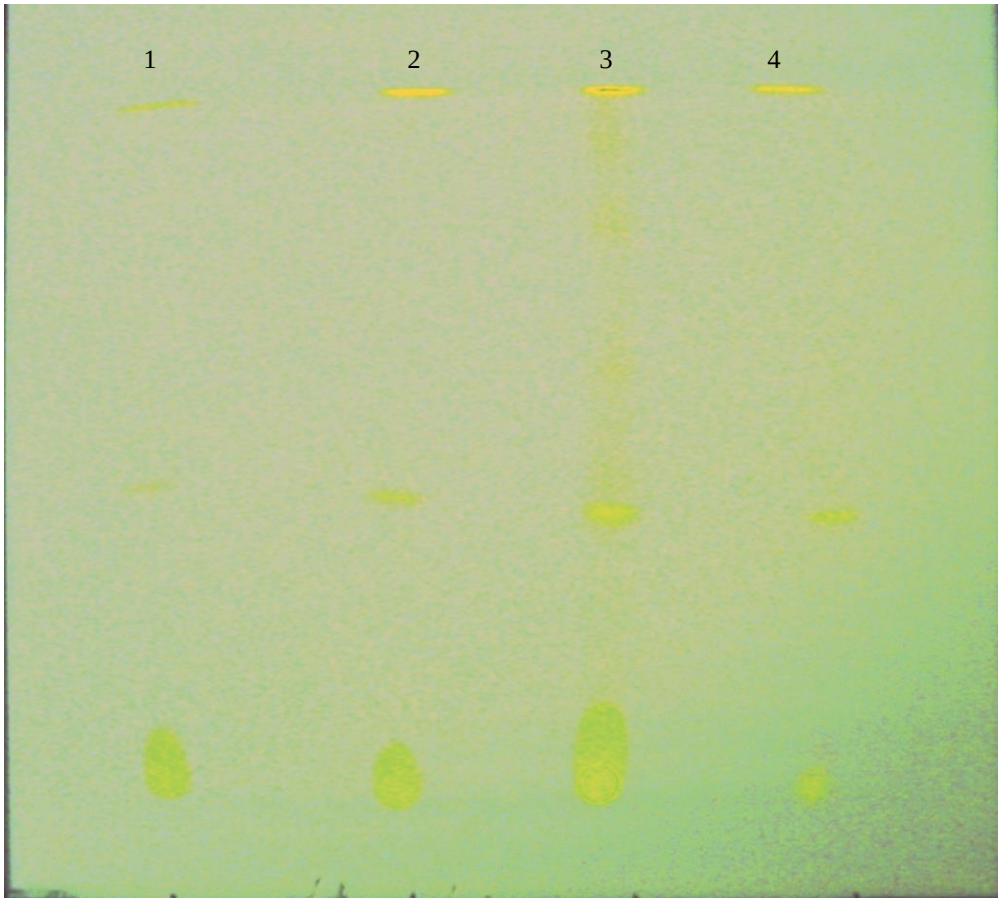


1 and 4= control 2 = treated with 43 $kgNha^{-1}$,
3 = treated with 86 $kgNha^{-1}$ 5= treated with 41 kg $P_2O_5ha^{-1}$.

Fig. 5. TLC separation of marigold flower carotenoids as affected by N and P fertilizers.

Effect of flower age on carotenoids

Fig. 6 shows the TLC separation of carotenoids extracted from marigold flowers of different ages. Flower age had significant effects on the qualitative characteristics of marigold carotenoids. The maximum number of separated carotenoids was obtained by the fully mature flower, which decreased in the over-mature stage.



1= Unopened flowers 2= newly opened flowers,
3= fully mature 4 = over mature flowers.

Fig. 6. TLC separation of marigold carotenoids extracted from flowers of different ages.

Marigold seed fixed oil

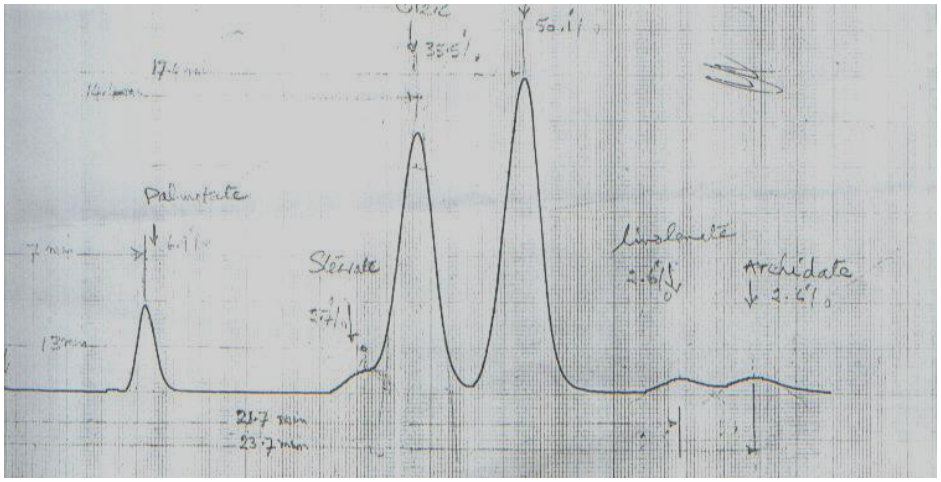
Table 4 shows the physico-chemical characteristics of marigold seed oil. Seed oil content was very low (1.47%), acid value was 0.85mgKOH/g, peroxide value was 3.40 mgq/kg, saponification value was 187 mgKOH/g and unsaturation index was 13.8%.

Table 4. Oil content and physico-chemical characteristic of marigold seed oil.

Oil	1.47%
Acid value	0.85mgKOH/g
Peroxide value	3.40 mgq/kg
U.I.*	13.80%
Saponification value	187 mgKOH/g

*Unsaturation index was calculated from the fatty acid composition determined by GLC by multiplying the percentage value for oleic, linoleic and linolenic by a factor of 1,2 and 3, respectively and summing up.

Fig. 7 shows the fatty acid composition of marigold oil determined by GLC. The major fatty acid was linoleic acid, approximating 50%, and was also characterized by the presence of arachidic acid. This may be useful in taxonomic studies of marigold.



1 = palmitic 2 = stearic 3 = oleic 4 = linoleic 5 = linolenic 6 = arachidic

Fig. 7. Typical GLC separation of methylesters of standard fatty acids.

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CONCLUSIONS

The following conclusions can be drawn from this study

1. Marigold flowers contained about 6 different carotenoids including lutein, the major component, and β-carotene.
2. When compared with salad rocket,reportedly lutein rich plant, most of the lutein of marigold flowers was present in the esterified form in contrast to rocket leaves in which case lutein was largely in the free form.
3. Nitrogen and phosphorus fertilizers did not affect flower carotenoids content.
4. Six carotenoids appeared in marigold flowers 15 days after flower opening and less types were observed in younger and older flowers.
5. The seeds of marigold contain 1.5% of fixed oil, the major fatty acid of which was linoleic acid.

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تأثير سماد النيتروجين والفسفور وعمر الزهرة على صبغة الكاروتين ومحتوى الزيت في زهرة الماريقولد

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الخلاصة

وادمدني، تمت زراعة بذور الماريقولد التي جمعت من نباتات زينة محلية في مزرعة كلية العلوم الزراعية، جامعة الجزيرة، الهدف من الدراسة هو بحث تاثير سماد النيتروجين والفسفور ومراحل نمو الزهرة على .في الرابع من مارس, 2007 السودان, كانت مستويات النيتروجين صفر، 43، 86 كجم (*Tagetes patula* L.)صبغة الكاروتين ومحتوى الزيت في زهرة الماريقولد قبل ان /هكتار. حصدت الازهار في مراحل نمو مختلفة وهي:P_2O_5 41 كجم هكتار ومستويات سماد الفسفور صفر,/نيتروجين تم استخدام تصميم القطاعات العشوائية الكاملة. أوضحت ومباشرة بعد تفتح الزهرة ومكتملة النضج وبعد 15 يوما من التفتح. تتفتح، نتائج التجربة أن المعاملة بالنيتروجين والفسفور أدت الى زيادة معنوية في طول النبات وعدد الافرع في نبات الماريقولد، ولكنها لم تؤثر على محتوى الكاروتينات الموجودة في أزهار الماريقولد كما أوضحت طريقة كروماتوغرافيا الطبقة الرقيقة أن أزهار الماريقولد تحتوي على حوالي 5 أنواع مختلفة من الكاروتينات بما فيها ليوتين وبيتاكاروتين. الليوتين الموجود في الماريقولد يوجد بصورة مرتبطة في شكل إستر مقارنة بالليوتين الموجود في الجرجير والتي يوجد فيها الليوتين بصورة حرة. ظهرت 5 أنواع من الكاروتينات في زهرة الماريقولد بعد 15 يوم من تفتح الأزهار، ووجدت نسبة أقل في الأزهار الأقل والأكبر عمراً. تحتوي بذور الماريقولد على1.5% من الزيوت الثابتة والمكون الأساسي لها من الأحماض الدهنية هو حمض اللينوليك.