Identification and Quantitative Estimation of Lutein in Iraqi *Spinacia oleracea* *Family Chenopodiaceae* by Using Chromatographic Methods

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Abstract:

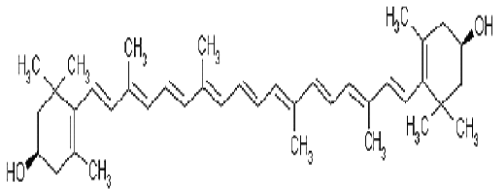
Lutein is an antioxidant carotenoid present in the highest quantities in dark, leafy green vegetables such as spinach. In this study, and for the first time we try to separate and calculate the quantity of lutein in Iraqi spinach to know the dietary requirements from this active drug to avoid age macula degeneration caused by lutein depletion.

Extraction and fractionation of lutein from Iraqi spinach leaves were carried out by soxhlet apparatus using petroleum ether: acetone (1:1) as a solvent system, then lutein was isolated by preparative thin layer chromatography and identified by thin layer chromatography using two different solvents system: (petroleum ether: diethyl ether:acetic acid) and (petroleum ether: acetonitrile: methanol) compared with standard , melting point, mixed melting point and high performance liquid chromatography (HPLC).50gm of Iraqi spinach gives about 32mg of lutein.

Key words:Lutein, carotenvid, spinacia plant.

Introduction:

The chenopodiaceae family consists of 100 species of evergreen or semievergreen annuals, perennials, and shrubs. Spinach is one of them; the leaves are the most frequently used parts of spinach[1,2]. spinach contains a number of antioxidant including carotenoids, polyphenols[3,4] and flavonoids (quercetin)[5]. The carotenoids are composed of 2 main classes, carotenes (betacarotene) and xanthophyllus (lutein)[6]. lutein (Fig1) is a powerful antioxidant that protect macula of the retina against damage by filtering blue light before it can damage the macula. If sunglasses are the first line of defense against blue light, lutein is the last[7,8].



Lutein;trans-lutein;4-[18-(4-hydroxy- 2,6,6-trimethyl-1-cyclohexenyl) -3,7,12,16-tetramethyl octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-3,5,5-trimethyl- cyclohex-2-en-1-ol[9]

Fig-1 structure of Lutein

Lutein provides nutritional support to our eyes and skin- the only organs of the body directly exposed to the outside environment. Lutein has been linked to promoting healthy of eye and skin through reducing the risk of

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macular degeneration[10,11], be good at protecting the eyes, the arteries and the lungs from damaging free radicals[12], support normal eye function and protect the retina by blocking harmful blue light[13] reduce the risk of heart diseases and cancers[14].

The amount of lutein in the eye may be depleting with age, and since our body doesn't make lutein, we must constantly replace it through the food we eat. Dark leafy green vegetables like spinach are especially good sources.

Many researches have suggested a minimum of 6-10mg per day of lutein is necessary to realize lutein's benefits[15] many studies around the countries investigate and calculate the amount of lutein in their spinach leaves (example in USA 58gm of fresh spinach contain about 6mg of lutein)[16,17], so it is our privilege to present this work to be the first phytochemical work that separate this active drug and calculate it's quantities in the Iraqi species.

Materials and Methods:

The plant material (leaves) of Iraqi *Spinacia oleracea* *Family Chenopodiaceae* was collected during the months of November and December from the garden of Pharmacy college and identified by department of pharmacognosy, college of pharmacy, University of Baghdad, and authenticated by National Iraqi Herbarium, Botany Directorate at Abu-Ghraib. The plant was dried at room temperature in the shade and was pulverized by mechanical mills and weighed.

50gm of the spinach leaves were extracted with 100ml of organic solvent (Petroleum ether : Acetone) (1:1) using soxhlet apparatus(Koyota , Japan) for 8 hours , then extract was evaporated to dryness under reduced pressure at a temperature not exceeding 40C° to give greenish colored residue (16.032gm)kept in closed dark container .

1. Thin Layer Chromatography (TLC):

The greenish residue was examined by TLC using the following system:-

Silica gel/ GF: a slurry was prepared by mixing 30gm silica gel GF (MERCK) with 60 ml distilled water. The slurry was spread out in a layer of 0.25mm thickness on 20X20 cm glass plates using "Jobling Laboratory Division TLC coater". The plates were activated at 110C° for one hour before use.

Developing solvent systems: the solvent mixture was placed in a glass tank (22.5cmX22cmX7.0cm) lined with Whatman No.1 filter paper.A 100ml of solvent mixture was placed in each tank and covered with a glass lid and allowed to stand for 45 minutes before use. Different solvent systems were used for carotenoid compounds[18] :

S1 Petroleum ether: Diethyl ether: Acetic acid[18]
80:20:1

S2 Petroleum ether: Acetonitrile: Methanol[18]
20:40:40

Standard References compound: pure Lutein compound (National Vitamin Company "Casa Grande")

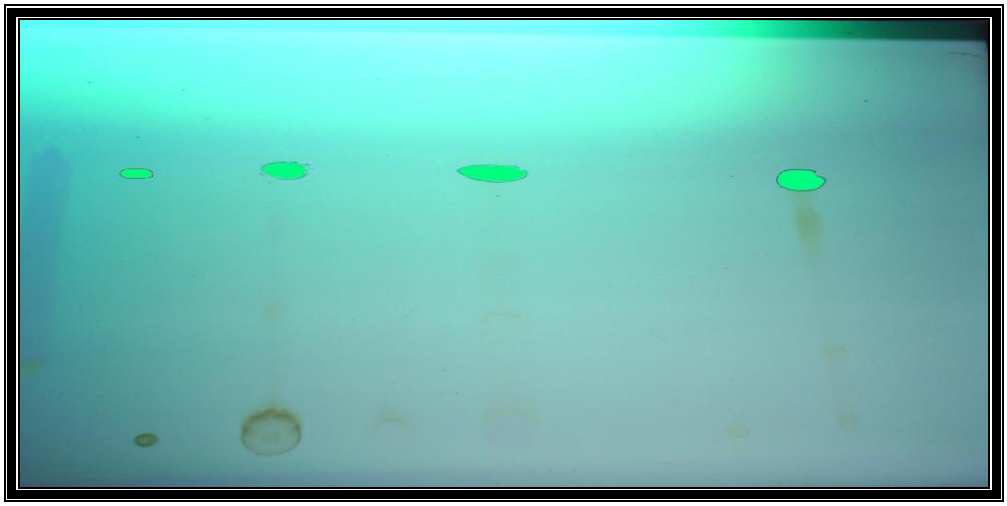
A) Detection method: the chromatogram was examined by eyes and under Ultra violet light (DESAGA HEIDELBERG) at wave length UV 445nm.

2.High Pressure Liquid Chromatography (HPLC)

HPLC equipped with a variable wave length detector, azorbax ODS 150mmX4.6mm id reverse phase column or equivalent, and a suitable 10-µ1 injection value. mobile phase : ethyl acetate: acetonitrile (12:88) 1.6 ml/min, injection volume 10µ1; detector wave length 450nm.

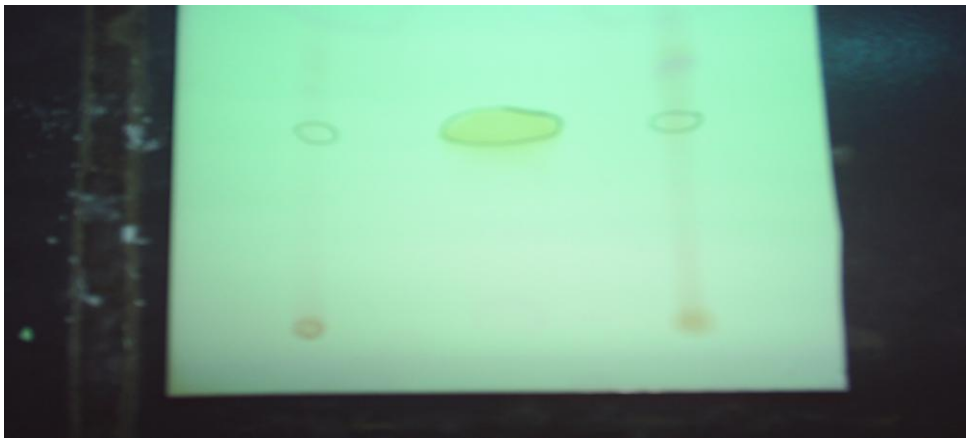
Results and Discussion:

Preliminary investigation indicated that Iraqi spinach leaves have lutein in a good amount, extraction done using mixture of two organic solvents since lutein is carotenoid pigment soluble in organic solvent. Initial identification of active compound (lutein) is done by thin layer chromatography using two different solvent systems, further purification and separation of Lutein from crude extract is done by preparative TLC. TLC plates of extract and standard showed the following characteristic spots: (Fig-2&3)



Standard Sample Standard Sample

Fig 2: TLC plate for the standard (lutein) and sample (spinach extract) using silica gel GF as a stationary phase and S1 as a mobile phase under 445nm wave length



Sample Standard Sample

Fig 3: TLC plate for the standard (lutein) and sample (spinach extract) using silica gel GF as a stationary phase and S2 as a mobile phase under 445nm wave length

From the previous figures we calculate the Retardation factor (R_f) value which is defined as the distance moved or traveled by the compound to the distance moved by the solvent and it is constant for each compound when the chromatography is carried out using the same technique, mobile phase, and the same conditions. Usually the R_f value is used for the identification of the separated compound by comparison with the R_f value of a standard**.**

Table 1:The Rf values of the extract and standard are tabled below.

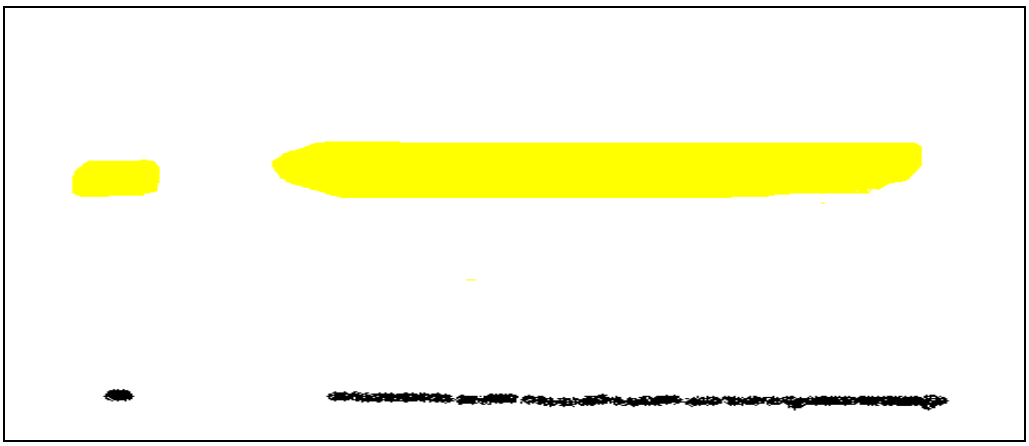
Solvent system	standard	extract
S1	0.53	0.51
S2	0.22	0.22

Separation and purification of lutein by preparative TLC

Preparative TLC plates were prepared by mixing of 75g silica gel with 150ml distilled water. The slurry was spread out in a layer of 2mm thickness on five 20X20cm glass plates. The plates were allowed to dry over night at room temperature and then activated by heating at 110C° for one hour before use.

The residue was dissolved in a minimum quantity of acetone and applied with standard reference lutein on a number of preparative TLC plates using (S1) solvent system. The solvent was allowed to rise to a height of 15cm from the base line. The band of lutein in the extract and standard lutein were observed under UV light at 445wave length (fig-4) both of them have the same Rf value.

Therefore lutein band had been scratched out, eluted with acetone, and then filtered. The filterate evaporated to dryness, in vacuo, to give yellow crystals (32mg), upon re-crystallization out of boiling ethanol 95% a fluffy yellow crystals were obtained having a sharp melting point of 176-177C°.



Standard **sample**

Fig 4: preparative TLC plate for the standard (lutein) and sample (spinach extract) using silica gel GF as a stationary phase and S1 as a mobile phase under 445nm wave length

Furthermore, the identity was approved by HPLC the retention time was also authenticated with standard reference as shown in (Fig-5&6).

Potentiation of the isolated Lutein together with the standard compound (Fig-7), show a sharp peak with higher intensity.

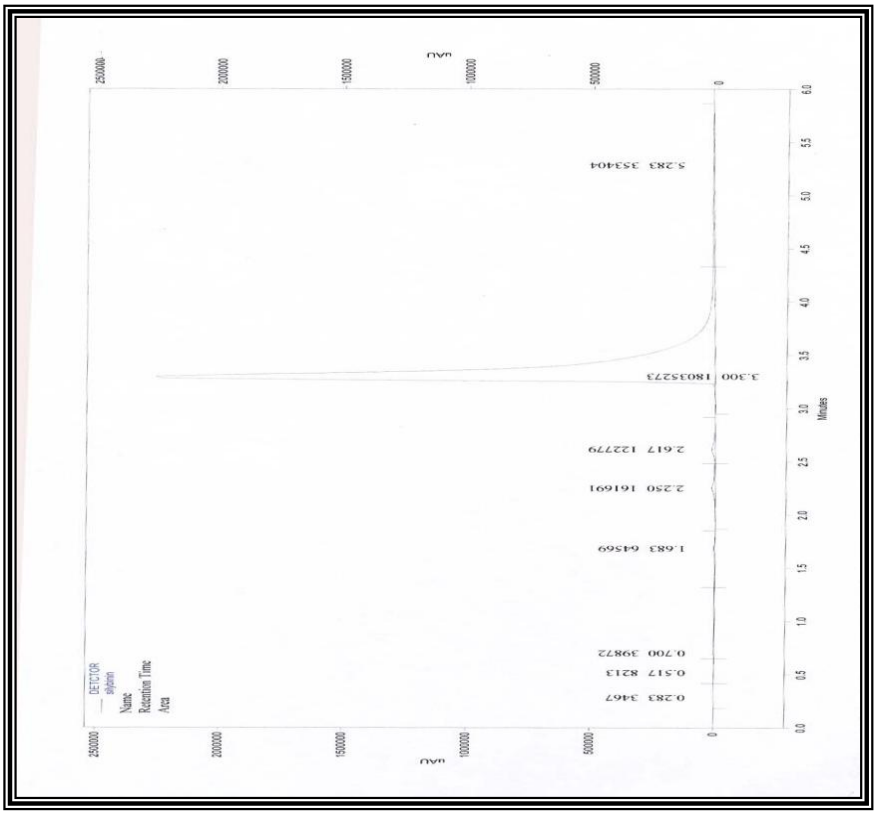


Fig 5: HPLC of standard Lutein

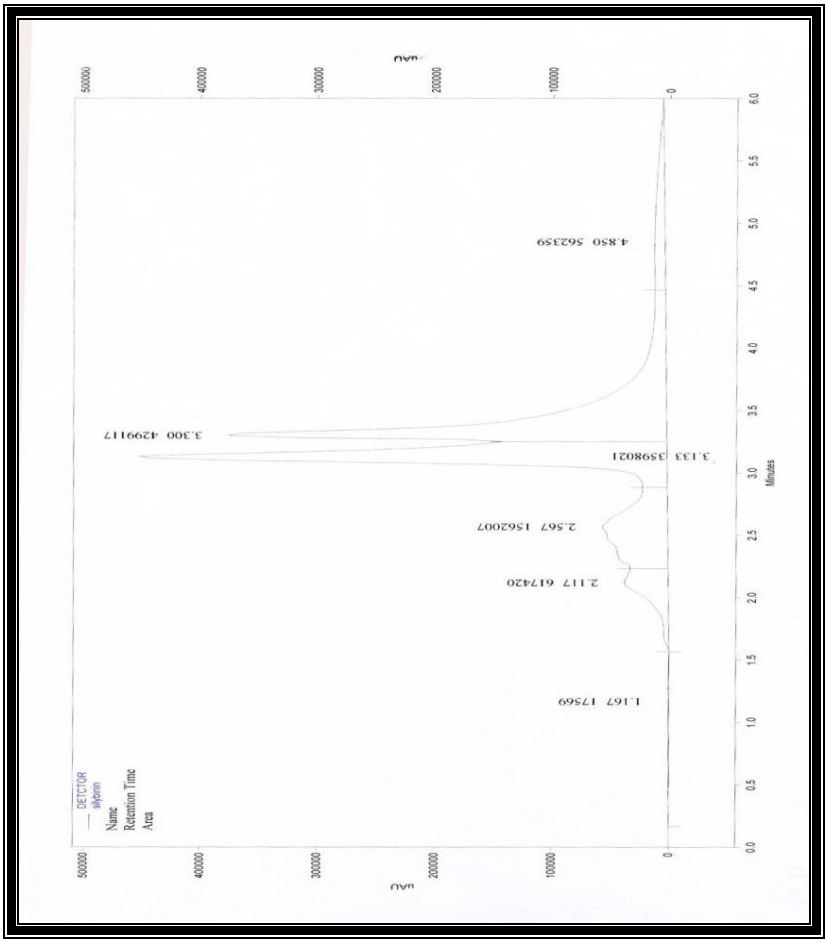


Fig 6: HPLC of extract

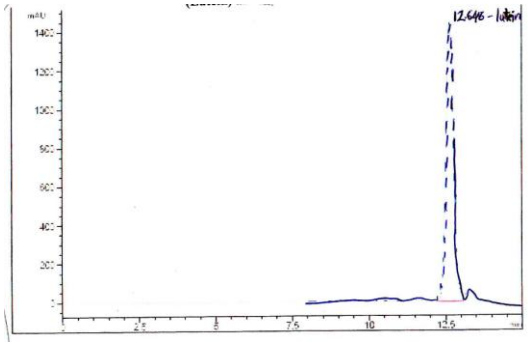


Fig 7:HPC for a mixture of equal quantities of the separated compound(Lutein) and standard reference

Lutein was separated from crude extract by preparative TLC as yellow pure crystals in a weight of 32mg from 50gm plant leaves,this resulte indicate that Iraqi spinach content of lutein is higher than that in other countries, since the American Optometric Association (AOA) reminds peoples that caring for eyes includes paying attention to nutrition and based on this research we can protect our eye from age-related eye diseases by eating this type of foods which is rich in lutein content specially Iraqi species which contain good quantity of lutein.(percentage of Lutein in Iraqi spinach :

50gm contain 32mg (0.032gm) so percentage will be

$\frac{0.032x100}{50}$= 0.064w/w

While percentage in a certain type of USA spinach

58gm contain 6mg [16,17] (0.006gm) so

Percentage will be $\frac{0.006x100}{58}$=0.01w/w

Conclusion:

Above result indicate that the Iraqi spinach contain lutein more than the other type and since the daily requirement from this important substance is 6-10mg per day[15] so Iraqi species provide a good source for Lutein to protect our eyes from age macula degeneration.

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التشخيص والتقدير الكمي للوتين في السبانغ العراقي باستعمال الطرق الكروماتوغرافية

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الخلاصة:

اللوتين هو صبغة كاروتينويدية مضادة للاكسدة وموجودة بكميات كبيرة في الخضروات الداكنة الخضرة مثل السبانخ اللوتين و المركبات المشتقة منه مثل زيزانثين تتركز في شبكية عين الانسان لحماية العين من الاذى الخارجي لكن مع تقدم عمر الانسان قد تصاب العين بما يسمى (هرمية الشبكية) فيبدا تركيز اللوتين بالانخفاض وفي هذه المرحلة يكون الانسان بحاجة ماسة الى هذه المادة اما عن طريق الغذاء او عن طريق الدواء لذا لاهمية هذه المادة للانسان فقد حاولنا في هذه الدراسة ولاول مرة في العراق فصل وحساب كمية اللوتين في السبانخ العراقي ومقارنته مع الاجناس الاخرى من السبانخ في انحاء اخرى من العالم.استخلاص وفصل اللوتين من اوراق السبانخ العراقي قد تم باستخدام جهار السوكسليت وباستخدام مزيج من البترول ايثر والاسيتون كمذيبات عضوية. ولقداثبتت لنا نتائج فحص المستخلص بطريقة كروماتوغرافيا الطبقة الرقيقة عن وجود مركب اللوتين الذي تم فصله عن بقية المركبات الموجودة في هذه النبتة على شكل بلورات صفراء نقية وذات درجة حرارة انصهارتبلغ 207-208م. ومن حساب كمية اللوتين الموجودة في جنس النبات العراقي اتضح انها تفوق الكمية الموجودة في اجناس اخرى لنفس النبات.