Flowers and Leaves of *Tropaeolum majus* L. as Rich Sources of Lutein

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ABSTRACT: As increasing evidence supports the role of lutein and zeaxanthin in reducing the risk of cataract and macular degeneration, food sources of these carotenoids are being sought. In the present study, the lutein content of the edible flowers and leaves of *Tropaeolum majus* L. was determined by high-performance liquid chromatography–photodiode array detector (HPLC-PDAD), complemented by HPLC-mass spectrometry (MS) for identification. Chemical reactions were also used as identifying parameters. The yellow and brownish orange flowers had 450 ± 60 μg/g and 350 ± 50 μg/g lutein, respectively. Violaxanthin, antheraxanthin, zeaxanthin, zeinoxanthin, β-cryptoxanthin, α-carotene, and β-carotene were also detected at very low levels. The leaves had 136 ± 18 μg/g lutein, 69 ± 7 μg/g β-carotene, 74 ± 23 μg/g violaxanthin, and 48 ± 13 μg/g neoxanthin. Lutein was partly esterified in the flowers and unesterified in the leaves. The flowers of *T. majus* are therefore excellent food sources of lutein and the leaves good sources of both lutein and the provitamin A β-carotene.

Keywords: *Tropaeolum majus* L., carotenoids, flowers, leaves, lutein

Introduction

Lutein and zeaxanthin make up the yellow pigment in the macula of the human retina (Bone and others 1988; Handelman and others 1988). Dietary intake and plasma levels of these carotenoids were found to have a statistically significant inverse relationship with the risk of macular degeneration (EDCC 1993; Seddon and others 1994; Snodderly 1995), the principal cause of irreversible blindness in elderly persons. There is also consistent evidence of a protective association between lutein in the diet and cataract (Moeller and others 2000). Thus, a search for sources of lutein and zeaxanthin is ongoing, and the need to know the contents of these 2 carotenoids in foods is widely acknowledged. Lutein is a dihydroxy derivative of α-carotene, and zeaxanthin is a dihydroxy derivative of β-carotene. These 2 carotenoids are not widely distributed in foods. In the extensive Brazilian database on food carotenoids (Rodriguez-Amaya 1999a), only green vegetables and some varieties of squash and pumpkin have appreciable amounts of lutein.

On the other hand, poultry feed must contain lutein or zeaxanthin because chicken selectively accumulates these dihydroxy carotenoids, which then color the egg yolk, the skin, and the muscle.

The current commercial source of lutein is the inedible marigold (*Tagetes erecta*) flower. Ingested lutein from marigold extract was shown to increase human macular pigment density, which reduces the risk of macular degeneration (Landrum and others 1997). Leafy vegetables are good to rich sources of lutein. Increased consumption of spinach and other greens was associated with a significant reduction in the risk of macular degeneration (Seddon and others 1994) and cataract (Hankinson and others 1992; Tavani and others 1996; Brown and others 1999; Chasan-Taber and others 1999).

In this work, the principal carotenoids of the flowers and leaves of *Tropaeolum majus* L. were quantified. *T. majus* (nasturtium) is an ornamental, annual, rapid-growing, bushy (about 30 cm tall) or vining (may extend up to 90 cm) plant. It has tender, rounded, blue-green, watercress-flavored leaves (5 to 15 cm across), held by long fleshy stalks, and showy trumpet-shaped yellow or orange flowers with reddish patches. The entire plant has a spicy peppery flavor. Leaves, flowers, and stems are used fresh in salads, and the green pods can be pickled and used as a substitute for capers. This herbal plant is believed to be medicinal, with antimicrobial, antimycotic, expectorant, and purgative properties, and is used for respiratory, ophthalmologic, and urinary tract infections. Native to Peru, it is now available throughout the world.

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Materials and Methods

Sample collection

The flowers were purchased from 3 supermarkets in the city of São Paulo (São Paulo, Brazil) in small packages of 7 g to 10 g. Because the leaves are not usually sold in markets, leaf samples were purchased in packages of about 20 g from one of the major producing farms in the state of São Paulo. The samples were collected at different times during the year.

For each sample lot, the yellow and the brownish orange flowers of 1 or 2 packages were separated, weighed (this amounted to 1 g to 5 g), and submitted to analysis. The leaves from 3 packages were homogenized in a food processor, and 2-g subsamples were weighed for analysis. The flowers and the leaves were analyzed without the stem.

Carotenoid analysis

The principal carotenoids of the flowers and leaves of *T. majus* were determined using a method developed for leaves (Kimura and Rodriguez-Amaya 2002) and also found to be appropriate for the flowers, from which carotenoids were easy to extract. This method consisted of isolating standards by open column chromatography and quantification by high-performance liquid chromatography (HPLC).

The flower sample was extracted with cold acetone using a mortar and pestle, which was found to be efficient in disintegrating small amounts of samples. Extraction and filtration on a Buchner or

sintered glass funnel was repeated until the residue was devoid of color (about 3 times), the total amount of acetone used being 300 mL. The carotenoids were partitioned to 100 mL petroleum ether and saponified overnight with an equal volume of 10% KOH in methanol. After washing, the carotenoid solution was concentrated in a rotary evaporator (T ≤ 30 °C) and brought to dryness under nitrogen. Immediately before injection into the liquid chromatograph, the carotenoids were redissolved in 2 mL of HPLC-grade acetone, 1 mL was filtered with a 0.22-μm PTFE syringe filter, and 10 μL was automatically injected into the HPLC equipment. The same procedure was followed for the leaves except that saponification was not carried out, considering that the hydroxy carotenoids were not esterified and the chlorophylls were well separated from the carotenoids in the HPLC chromatogram. Quantification was carried out by external standardization.

Identification of the carotenoids was done according to Rodriguez-Amaya (1999b). This involved the combined use of retention times, co-chromatography with authentic carotenoids, visible absorption spectra (λ_{max} and spectral fine structure) obtained with a recording spectrophotometer (Beckman DU 640, Beckman Instruments, Inc., Fullerton, Calif.) and with the photodiode array detector, and chemical tests for the xanthophylls. Spectral fine structure was expressed as %III/II, the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the 2 peaks as baseline, multiplied by 100 (Britton 1995). Chemical reactions such as acetylation with acetic anhydride of secondary hydroxyl group, methylation with acidified methanol of allylic secondary hydroxyl group, and epoxide-furanoid rearrangement of 5,6-epoxy groups were also carried out. The progress of the reaction was monitored spectrophotometrically and/or by thin layer chromatography (TLC) on silica gel plates developed with 5% methanol in toluene.

For the chemical tests and for the isolation of the standards, the carotenoids were isolated on MgO:Hyflosupercel (1:2, activated for 4 h at 110 °C) packed to a height of 20 cm in 2.5-cm inner dia × 30-cm glass columns. This involved extraction of a 50-g sample of flower or leaf, partition, and saponification as described previously. The washed extract was concentrated in a rotary evaporator and applied on the column. Because of the much higher concentration of lutein, 2 columns were used for the flower. In the 1st column, α-carotene was eluted with petroleum ether, and β-carotene, zeinoxanthin, β-cryptoxanthin, lutein, antheraxanthin, and violaxanthin by 4% ethyl ether, 4% acetone, 10% acetone, 18% acetone, and 40% acetone (for the last 2 carotenoids) in petroleum ether, respectively. The lutein fraction was rechromatographed in another column with larger dimension (3.5-cm inner dia × 30 cm) on which lutein and zeaxanthin were separated and eluted with 18% acetone in petroleum ether. Standards for quantification were obtained from parsley. For these standards and for the identification of the carotenoids of the leaves of *T. majus*, β-carotene, violaxanthin, lutein, and neoxanthin were eluted from the MgO:Hyflosupercel column by 4% ethyl ether, 12% to 16% acetone, 18% acetone, and 40% acetone in petroleum ether, respectively. Separation on the column was monitored visually.

The average purity of the standards, calculated as the percentage of the carotenoid's peak area relative to total area, was 92% for neoxanthin, 97% for violaxnthin, 93% for lutein, and 90% for β-carotene. The concentrations of the standard solutions were corrected accordingly.

The HPLC chromatographic system consisted of a Waters separation module (model 2690), equipped with an autosampler injector and a UV-visible photodiode array detector (PDAD) (Waters model 996, Water Corp., Milford, Mass.), controlled by a Millenium workstation (version 2010, Water Corp., Milford, Mass.). Detection for quantification was at the wavelengths of maximum absorption (max plot), that is, 441 nm for violaxanthin, 448 nm for lutein, 454 nm for β-carotene, and 439 nm for neoxanthin. Reversed-phase chromatography was carried out using a monomeric C_{18} column (Spherisorb ODS2, Water Corp., Milford, Mass., 3 μm, 4.6 × 150 mm). The mobile phase consisted of acetonitrile containing 0.05% triethylamine, methanol, and ethyl acetate, used at a flow rate of 0.5 mL/min. A concave gradient (curve 10) was applied from 95:5:0 to 60:20:20 in 20 min, maintaining this proportion until the end of the run. Reequilibration took 15 min.

The electron impact mass spectrum of lutein was obtained with a Waters Integrity System equipped with a Thermabeam HPLC–MS (Water Corp., Milford, Mass.) interface, the temperatures of the expansion region and nebulizer being 80 °C and 90 °C, respectively. The ionizing voltage was 70 eV, and the temperature of the ion source was 210 °C. The *m/z* range was 150 to 650.

Results and Discussion

Eight carotenoids were identified in the *T. majus* flowers: violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), antheraxanthin (5,6-epoxy-5,6-didehydro-β,β-carotene-3,3′-diol), lutein (β,ϵ-carotene-3,3′-diol), zeaxanthin (β,β-carotene-3,3′-diol), zeinoxanthin (β,ϵ-carotene-3-ol), β-cryptoxanthin (β,β-caroten-3-ol), α-carotene (β,ϵ-carotene), and β-carotene (β,β-carotene). The identifying properties of these carotenoids are presented in Table 1 and discussed subsequently.

The carotenoid identified as violaxanthin had a visible absorption spectrum with well-defined spectral structure (%III/II = 98), typical of a carotenoid with 9 conjugated double bonds in the polyene chain. Positive acetylation and the chromatographic behavior (t_R = 9.4 min, R_F = 0.12) demonstrated the presence of 2 hydroxyl groups whereas the epoxide-furanoxide rearrangement (hypsochromic shift of 40 nm) proved the existence of 2 epoxide groups at the 5,6 and 5′,6′-positions.

The visible spectrum of antheraxanthin, with λ_{max} at slightly higher wavelengths than those of violaxanthin and less fine structure (%III/II = 60), was consistent with a carotenoid having 9 of 10 conjugated double bonds in the polyene chain and 1 in a ring. The presence of 2 secondary hydroxyls was manifested by the chromatographic behavior (t_R = 12.6 min, R_F = 0.15) and the positive reaction to acetylation, the non-allylic position being shown by the negative response to methylation. That a 5,6-epoxide was also present was demonstrated by the hypsochromic shift of 20 nm on addition of dilute HCl.

Lutein showed the same visible spectrum of antheraxanthin, with λ_{max} and fine structure (%III/II = 60) in accordance with a chromophore of 10 conjugated double bonds, 9 in the polyene chain and 1 in a β-ring. The presence of 2 secondary hydroxyls was confirmed by the positive reaction to acetylation and the chromatographic behavior (t_R = 15.9 min, R_F = 0.21), the allylic position of 1 of them being shown by the positive response to methylation, forming a monohydroxylated carotenoid.

The identification of lutein was confirmed by HPLC-MS. The mass spectrum showed the molecular ion prominently at *m/z* 568, consistent with $C_{40}H_{56}O_2$, and characteristic fragments at *m/z* 550 $[M-18]^{+\cdot}$ and at *m/z* 532 $[M-18-18]^{+\cdot}$, corresponding to the loss of 1 and 2 molecules of water, respectively. Other peaks were observed at *m/z* 476 $[M-92]^{+\cdot}$, due to the elimination of toluene from the polyene chain, *m/z* 430 $[M-138]^{+\cdot}$ and *m/z* 338 [M-toluene-138]$^{+\cdot}$ in which 138 corresponded to either the ϵ- or β-end group of lutein.

Zeaxanthin presented a visible spectrum with λ_{max} higher than

Table 1—Wavelengths of maximum absorption and response to chemical test of the carotenoids of *Tropaeolum majus* L. flower[a]

Peak	Identification	λ_{max} (nm)[b]	λ_{max} (nm)[c]	%III/II	Response to chemical tests
1	Violaxanthin	417, 441, 470	416, 440, 468	98	Positive to 5,6-epoxy test (2 groups) Positive to acetylation (2 OH groups)
2	Antheraxanthin	423, 447, 474	421, 443, 471	60	Positive to 5,6-epoxy test (1 group) Positive to acetylation (2 OH groups)
3	Lutein	423, 448, 475	421, 443, 472	60	Positive to acetylation (2 OH groups) Positive to methylation (1 allylic OH)
4	Zeaxanthin	(428), 454, 480	(425), 448, 476	25	Positive to acetylation (2 OH groups) Negative to methylation
5	Zeinoxanthin	423, 447, 475	421, 443, 472	60	Positive to acetylation (1 OH group) Negative to methylation
6	β-Cryptoxanthin	(426), 454, 481	(425), 448, 476	25	Positive to acetylation (1 OH group) Negative to methylation
7	α-Carotene	425, 448, 476	422, 444, 472	58	No substituent
8	β-Carotene	(428), 454, 480	(424), 448, 476	25	No substituent

[a]Parentheses indicates a shoulder; %III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the 2 peaks as baseline, multiplied by 100.
[b]λ_{max} (nm) in the mobile phase, obtained by photodiode array detector (PDAD).
[c]λ_{max} (nm) in petroleum ether.

those of lutein and little definition of the peaks (%III/II = 25), commensurate with a chromophore of 11 conjugated double bonds, 2 of which situated in rings. Acetylation and the chromatographic behavior (t_R = 17.1 min, R_F = 0.19) confirmed the presence of the hydroxyl groups, the non-allylic position of which was shown by the negative response to methylation.

Zeinoxanthin showed a visible spectrum similar to those of antheraxanthin and lutein, consistent with a carotenoid of 10 conjugated double bonds, 1 situated in a β-ring. The presence of a hydroxyl group in a non-allylic position was reflected by the positive response to acetylation and negative methylation, and the chromatographic behavior (t_R = 24.4 min, R_F = 0.56).

β-Cryptoxanthin, has the same chromophore as zeaxanthin, with 11 conjugated double bonds, 2 of which located in β-rings, and thus the same visible spectrum. The existence of a non-allylic hydroxy substituent was demonstrated by the chromatographic behavior (t_R = 26.3 min, R_F = 0.44) and by the positive reaction to acetylation and negative response to methylation.

Not having functional groups, diagnostic chemical reactions are not done with carotenes and the identification is based primarily on the chromatographic behavior and the λ_{max} and fine structure of the visible spectrum.

As lutein, α-carotene had a visible spectrum manifesting a conjugated double bond system with 9 double bonds in the polyene chain and 1 in a β-ring. β-Carotene exhibited a visible spectrum with λ_{max} higher than those of α-carotene and much less spectral fine structure, commensurate with a chromophore of 11 conjugated double bonds, 2 of which situated in rings. The absence of functional groups was shown by the chromatographic behavior (t_R = 36.3 min and R_F = 0.99 for α-carotene, t_R = 37.3 min and R_F = 0.99 for β-carotene).

Because inconclusive or incorrect identifications can be noted in the literature, Pfander and others (1994) and Schiedt and Liaaen-Jensen (1995) recommended that the following minimum criteria for identification be fulfilled: (1) the visible (or ultraviolet for shorter chromophores) absorption spectrum (λ_{max} and fine structure) in at least 2 different solvents must be in agreement with the chromophore suggested; (2) chromatographic properties must be identical in at least 2 systems, preferably TLC (R_f) and HPLC (t_R) and co-chromatography with an authentic sample should be demonstrated; and (3) a mass spectrum should be obtained, which allows at least the confirmation of the molecular mass. However, the requirement of a mass spectrum would limit carotenoid analysis to a very few laboratories around the world, precluding its execution in areas where it is probably most needed. We have shown in the case of lutein in this work and other carotenoids in previous studies (Mercadante and others 1997, 1998; Azevedo-Meleiro and Rodriguez-Amaya 2004) that identifications based on the chromatographic behavior, visible spectra and chemical tests (for xanthophylls) were all confirmed by the mass spectra. The judicious and combined use of these identifying parameters can conclusively identify carotenoids with known structures. Mass spectrometry is indispensable for the elucidation of the structures of unknown carotenoids. Erroneous identification in the literature can be observed when the retention time/co-chromatography is used as the only basis for identification or in the case of xanthophylls, only retention time and the visible spectrum. On the other hand, the mass spectrum, especially when some of the characteristic fragments are missing, cannot be used as the sole criterion for identification.

The typical chromatograms of the carotenoids of saponified and unsaponified samples of *T. majus* flowers are shown in Figure 1 and 2, clearly demonstrating that lutein is partly esterified. The predominance of lutein is also shown, comprising 65% to 70% of the total carotenoid content. Interestingly, zeaxanthin, the other carotenoid found in the macula, had higher level than the other minor carotenoids.

The yellow flowers had 450 ± 60 μg/g of lutein while the orange flowers had 350 ± 50 μg/g lutein (Table 2), indicating that the deep orange color of *T. majus* flower would not be due to a high concentration of the yellow lutein. Indeed, we noted water-soluble orange and red pigments, which could not be partitioned to petroleum ether, in this flower. This is contrary to what was observed in marigold, in which the lutein content increased from the yellow to the dark orange flowers (Gregory and others 1986).

The average weight of the flower was 0.6 g. Thus, 1 yellow flower would provide 270 μg of lutein and 1 orange flower 210 μg lutein. These high values make the *T. majus* flower an excellent functional food.

Gregory and others (1986) reported that the concentration of

Table 2—Carotenoid content of *Tropaeolum majus* L. flowers and leaves

Carotenoid	Concentration (μg/g)[a]		
	Yellow flower	Orange flower	Leaves
Neoxanthin	ND[b]	ND[b]	48 ± 13
Violaxanthin	Trace	Trace	74 ± 23
Lutein	450 ± 60	350 ± 50	136 ± 18
β-Carotene	Trace	Trace	69 ± 7

[a]Means and standard deviations of 6 sample lots collected at different times during the year.
[b]Not detected.

lutein ester in fresh marigold varied from 4 μg/g in greenish yellow flowers to 790 μg/g in orange brown flowers. Piccaglia and others (1998) found that the total lutein content (free lutein + lutein esters) in the petals of 10 types of marigold belonging to the species *Tagetes patula* and *Tagetes erecta* ranged from 170 to 5700 μg/g. The lutein content of *T. majus* falls within these ranges.

Figure 3 shows a typical chromatogram of an unsaponified sample of the *T. majus* leaves, showing that lutein and the other hydroxy carotenoids are not esterified. Unlike flowers and fruits, which vary in the carotenoid composition qualitatively and quantitatively, leaves have been consistently shown to have the same carotenoid pattern, the principal carotenoids being lutein, β-carotene, violaxanthin, and neoxanthin. This was again shown in the present study. The 1st 3 carotenoids were identified by the same identifying parameters as described previously. Neoxanthin presented a visible spectrum (λ_{max} in PE = 414, 438, 466 nm; λ_{max} in the mobile phase = 415, 439, 467 nm) with defined spectral fine structure (% III/II = 88), consistent with a chromophore of 8 conjugated double bonds and an allene group. The presence of 3 hydroxyl groups, indicated initially by the chromatographic behavior (t_R = 7.7 min, R_F = 0.07), was confirmed by the positive response to acetylation. The 5,6- to 5,8-epoxide rearrangement (hypsochromic shift of 20 nm) reflected the existence of a 5,6- epoxide.

˜The concentrations of the principal carotenoids of the leaves are presented in Table 2. These levels are much higher than those found in common commercialized leafy vegetables (Ramos and Rodriguez-Amaya 1987; Mercadante and Rodriguez-Amaya 1991; Rodriguez-Amaya 1999a; Kimura and Rodriguez-Amaya 2003).

Conclusions

The edible *T. majus* flower is an excellent source of lutein, the yellow flowers having higher levels than the dark orange flowers. The leaf is a good source of lutein and the provitamin A β-carotene. More studies are warranted to promote the use of these materials as functional food.

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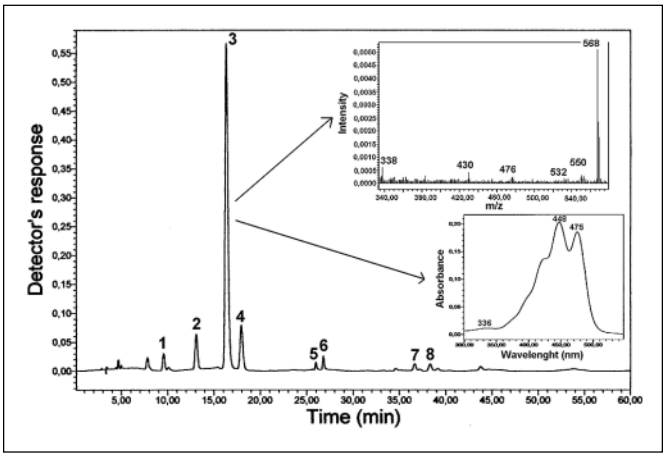

Figure 1—Typical high-performance liquid chromatography (HPLC) chromatogram of the carotenoids of a saponified sample of *Tropaeolum majus* L. flowers. Peak identification: 1 = violaxanthin, 2 = antheraxanthin, 3 = lutein, 4 = zeaxanthin, 5 = zeinoxanthin, 6 = β-cryptoxanthin, 7 = α-carotene, 8 = β-carotene. Inset: mass and visible absorption spectra of lutein.

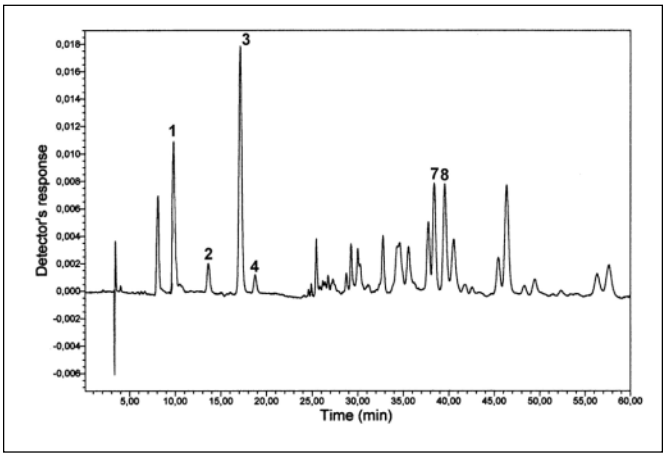

Figure 2—Typical high-performance liquid chromatography (HPLC) chromatogram of the carotenoids of an unsaponified sample of *Tropaeolum majus* L. flowers. Peak identification: 1 = violaxanthin, 2 = antheraxanthin, 3 = lutein, 4 = zeaxanthin, 7 = α-carotene, 8 = β-carotene. Peaks in the monohydroxy and carotene region are monoesters and diesters, respectively.

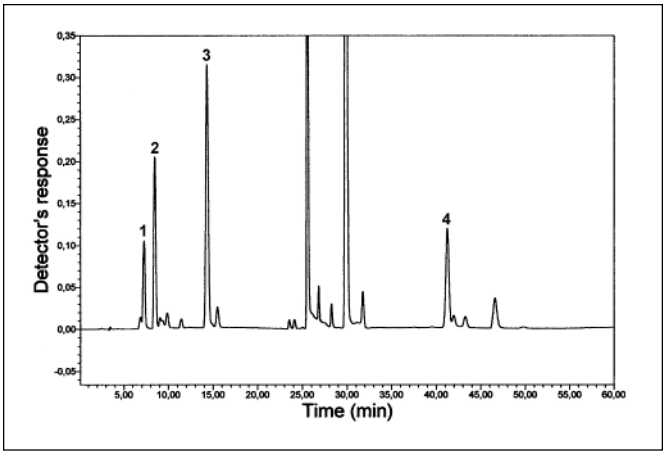

Figure 3—Typical high-performance liquid chromatography (HPLC) chromatogram of an unsaponified sample of *Tropaeolum majus* L. leaves. Peak identification: 1 = neoxanthin, 2 = violaxanthin, 3 = lutein, 4 = β-carotene. The other principal peaks are those of chlorophylls.

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