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Comparison of Antioxidant Activity of α-, β-Carotene, Lutein and Lycopene by High Pressure Liquid Chromatography

Kim(Jun), Hyeyoung

Div. of Biochemical Pharmacology, Korea Ginseng & Tobacco Research Institute, Taejon, Korea

ABSTRACT

A new HPLC method for determining malonaldehyde content in lipid peroxidation systems was developed and antioxidant activities of α-, β- carotene, lutein and lycopene were compared by a newly developed HPLC analysis and by TBA value. In addition, malonaldehyde forming ability of rat liver microsome was determined depending on thawing numbers. As results, malonaldehyde was eluted at a retention of 5.60 min and showed a linear relationship between peak area and concentration in standard curve. The MA content of microsome decreased with thawing numbers possible by destruction of cellular membranes. Lycopene, lutein and α-carotene showed stronger antioxidant activities than β-carotene or DL-α-tocopherol both in Fe^{+3}-ADP/NADPH and in paraquat/NADPH system. The inhibitory effects of carotenoids and DL-α-tocopherol on Fe^{+3}-ADP/NADPH lipid peroxidation system was similar by TBA value and by the HPLC analysis for malonaldehyde.

KEY WORDS : antioxidant activity · carotene · lutein · lycopene · HPLC.

INTRODUCTION

In the past the biological activity of β-carotene was considered limited to its provitamin A role. Recent evaluations of its biological importance emphasized β-carotene function as a potent singler oxygen scavenger and antioxidant[1)2)]. Because antioxidants have substantial potential to inhibit carcinogenesis and other adverse processes related to oxidative events[3~5)], β-carotene is considered to be biologically and clinically important agent. Studies on bacteria, Salmonella typhimurium (TAIOO)[3)] and animal cells such as Chinese hamster ovary(CHO) cells[4)] and 10T 1/2 cells[5)] exposed to either ultraviolet light or by a combination of chemical carcinogens and ultraviolet light indicated that β-carotene can function as a antimutagenic compound or can prevent malignant transformation. Most of the animal studies support that dietary carotenoids can prevent chemically or UV light/X-ray induced-skin tumor development[6~9)] and possibly act as immunoenhancing agents by preventing the spread of developing tumors[10)11)]. The mechanism of action of carotenoids for these chemopreventive actions is considered as the ability of these compounds to quench electronically excited species or quench radical reactions[10)11)]. Burton and Ingold[12)] suggested that β-carotene might interact with the peroxy radical species(LOO ·) in lipid peroxidation, thus inhibiting the propagation step of lipid peroxidation.

Malonaldehyde(MA) is one of the most studied

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products of lipid peroxidation and has been implicated in aging, mutagenesis, and carcinogenesis. The toxicity of MA is believed to be the result of its reactivity with biological nucleophiles such as amino acids and thiols and its ability to induce cross-linking of proteins and nucleic acids[13]. Several methods have been developed to estimate MA formed by lipid peroxidation. The most widely used method is thiobarbituric acid(TBA) assay. However, TBA method is not specific for MA and often overestimates MA levels because some chemicals, such as aldehydic compounds, react with TBA to produce absorbance spectra similar to those of the TBA-MA complex. Besides the lack of specificity, artificial production of MA could be occurred by heat and acid in the TBA reaction itself[14)15)].

In the present study, a new HPLC method for measuring MA content of lipid peroxidation systems was developed to obtain a shorter retention time. Antioxidant activities of carotenoids such as α-carotene, β-carotene, lutein and lycopene, which have slightly different structures, were compared both by the newly developed HPLC analysis and by TBA value. For lipid peroxidation experiments, liver microsomes were frozen at −20℃ and used after thawing. Therefore, MA forming ability of liver microsome depending on thawing numbers was also compared.

MATERIALS AND METHODS

Materials

The materials used in the study were obatined from the following sources : paraquat dichloride, nicotinamide adenine dinucleotide phosphate(NADPH), adenosine 5'-diphosphate(ADP), DL-α-tocopherol, α-carotene, β-carotene, lutein, lycopene, bovine serum albumin(BSA), 2-thiobarbituric acid(TBA) and 1,1, 3,3-tetraethoxypropane from Sigma chemical Co.(St. Louis, MO) ; Tris (hydroxymethyl) aminomethane, Kanto chemical Co.(Tokyo, Japan) ; Trichloroacetic acid, Junsei chemical Co.(Tokyo, Japan) ; ferric chloride from Fluka chemical Co.(Switzerland) ; acetonitrile for HPLC, J.T. Baker chemical Co.(Phillipsburg, NJ) and membrane filter(GA-8, TF-200 ; 0.2μ) from Gelman sciences Inc.(Ann Arbor, MI). All other reagents were of guaranteed reagent grade commercially available. The structures of carotenoids used in the study are shown in Fig. 1.

Calibration and preparation of the malonaldehyde standard

A malonaldehyde stock solution(10mM) was prepared from malonaldehyde tetraethyl acetal(1,1, 3, 3-tetraethoxypropane). One millimole(220mg) of the acetal was dissolved in 100ml of 1% (v/v) sulfuric acid. After 2hr standing at ambient temperature, 1ml of the solution was brought to a volume of 100ml with 1% (v/v) sulfuric acid and the malonaldehyde concentration was checked by measuring the UV absorbance at 245nm(ε=13, 700). For HPLC calibration curve aliquots of a

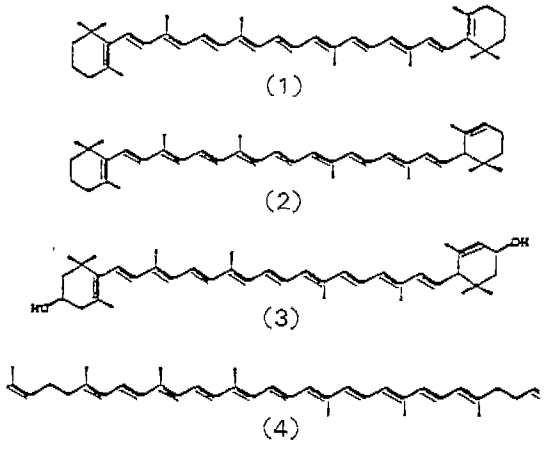

Fig. 1. Structures of carotenoids used in the study (1) β-carotene (2) α-carotene (3) lutein (4) lycopene.

10 mM stock solution was diluted with 0.1M Tris buffer(pH7.4) to give a final malonaldehyde concentration in the range of that present in the peroxidized microsomal suspension, preferably 20.0×10^{-6}M.

HPLC system and condition

Dionex basic chromatography module(Dionex Co., Sunnyvale, CA) connected with SP4270 computing integrator(Spectra-physics Co., San Jose, CA) and Applied Biosystems 1000s diode array detector(Ramsey Analytical Div., San Jose, CA) was used. The column was a Lichrosorb NH_2 column (10μm particle size ; E. Merck, Darmstadt, F.R. Germany). A sample of microsomal suspension or malonaldehyde standard solution was serially filtered with a membrane filter(0.45μm and 0.2μm) and injected into HPLC with a 50μl Hamilton syringe(Hamilton Co., Reno, Nevada). HPLC chromatographic condition is shown in Table 1.

Preparation of liver microsome

Livers from male Sprague-Dawley rats(200~250 g) were homogenized and centrifuged at 1,500xg for 20min in a refrigerated centrifuge. The supernatant was further centrifuged at 20,000xg for 10 min and 120,000xg for 60min to harvest microsomal fraction. The pellet was suspended in 0.1M Tris-HCl buffer, pH7.4[16]. Protein concentration of microsomal suspension was measured by the method of Lowry et al[17].

Table 1. HPLC condition

Condition	Malonaldehyde analysis
Column	Merck Lichrosorb NH_2 column (10μm, 25cm×4.0mm)
Mobile phase	0.1M pH7.4 Tris buffer/acetonitrile (9 : 1, v/v)
Flow rate	1ml/min
U.V. detection	270mn
Pressure	900psi
Temperature	ambient

In vitro microsomal lipid peroxidation

Two kinds of enzymatic lipid peroxidation systems were used : Fe^{+3}-ADP/NADPH system and paraquat/NADPH system. The former system contained microsomal protein(1mg/ml), 1.7mM ADP, 0.1mM $FeCl_3$, 0.1mM NADPH in 0.1M Tris-HCl buffer(pH7.4) and 100μM carotenoids or DL-α-tocopherol dissolved in absolute ethanol. Incubations were carried out at 25℃ for 30min[16]. For the latter system, reaction mixtures containing 1mg protein/ml of microsome, 0.2mM paraquat, 0.5mM NADPH, 150mM KCl in 0.1M Tris-HCl buffer, pH7.4 and 100μM carotenoids or DL-α-tocopherol in ethanol were incubated at 37℃ for 1hr[18]. Lipid peroxidation was determined by the formation of the TBA-reactive material, malondialdehyde (MDA) using a method of Fairhurst et al[16]. For malonaldehyde(MA) determination by HPLC, microsomal suspensions after incubation were filtered and directly injected to HPLC system.

RESULTS AND DISCUSSION

HPLC procedure for standard curve of MA

The spectrum of MA stock solution showed a maximum absorption at 270nm, which was in agreement with Esterbauer et al[19]. They detected MA at a retention time of 7min but MA was eluted at 5.6min in the present system(Fig. 2) by simply changing the concentration of the eluent which was also used in the system of Esterbauer et al[19]. The stock solution of MA was diluted with 0.1M Tris-HCl buffer, pH7.4 to achieve a final concentration in the range 5.0×10^{-6} to 75.0×10^{-6}M for the preparation of the standard curve(Fig. 3). There was a linear relationship between peak area and MA concentration. Recovery of standard MA in this procedure, after the addition to the known

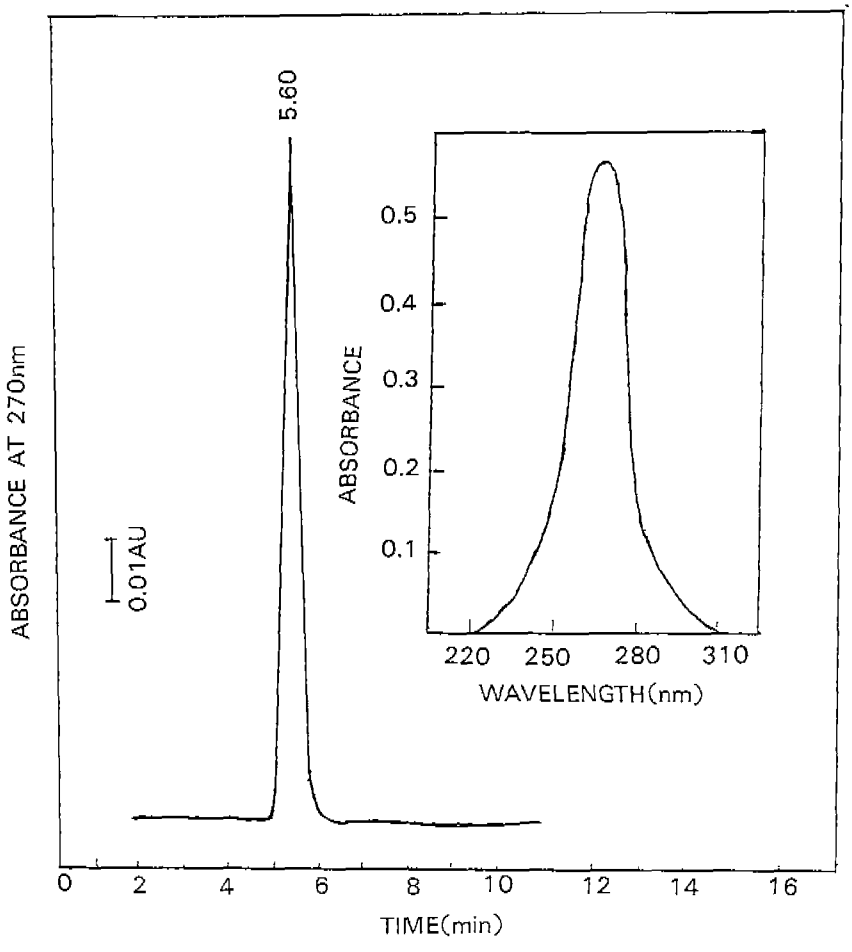

Fig. 2. HPLC elution profile for standard malonaldehyde(MA), which was prepared from tetraethoxypropane. Retention time is given above the peak in minutes. Inset shows the on-line spectrum of the MA as taken during its elution from the HPLC column ; the absorption maximum is indicated at 270nm. AU indicates an Absorbance Unit.

concentrations similar to those present in the microsomal suspension, was above 96%.

Csallany et al[20] reviewed HPLC methods for the quantification of MA. They found that none of these methods satisfied the need for a simple, sensitive and specific method for quantification of bona fide MA in tissues because of the requirement of a large amount of tissue, low recovery or unidentification of the questioned peak. Bird et al[21] attempted to separate TBA-MA complex but could not eliminate the possibility of some other TBA-aldehyde complex eluting with TBA-MA complex. Csallany et al[20] separated free MA in biological systems specifically but retention time was too long as 49.9min. Recently TBA-MDA(malondialdehyde) adduct was quantitated with a fluorescence detector and picomole quantities of MDA in plasma and liver samples were detected with 4.9min[22]. Even though the lower detection limit of MA in present system has not been checked, the present HPLC method can be easily applied for the detection of MA in biological systems when

fluorescence detection is not available.

MA forming ability of liver microsome depending on thawing times

After 30min-incubation in Fe^{+3}-ADP/NADPH system, liver microsomes were peroxidized and peak area of MA was increased at a retention of 5.60min(Fig. 4). Earlier peak was identified as nicotinamide(3.14min) present both in normal microsome and reaction mixtures while later peak could not be identified but surmised as a reaction product of lipid peroxidation other than MA. MA concentration in liver microsome was significantly decreased with thawing numbers(Table 2). This result suggested that repeated thawing may destroy microsomal membranes, thus the process of lipid peroxidation from cellular membranes including endoplasmic reticulums might be decreased.

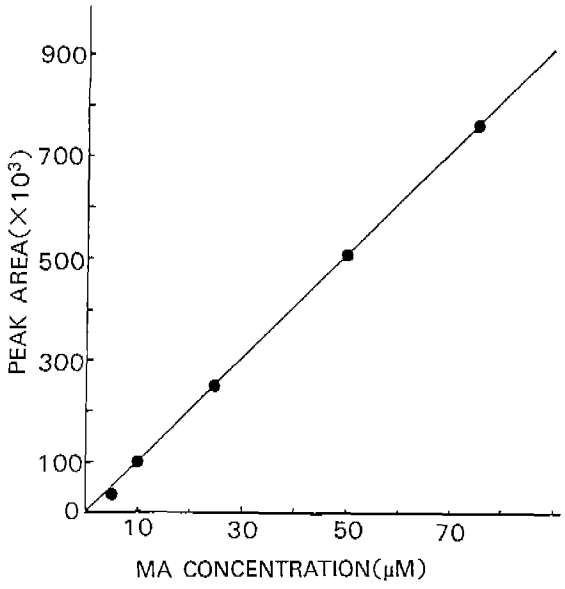

Fig. 3. Standard curve for HPLC of malonaldehyde (MA) on a Lichrosorb NH_2 column : Peak area vs injected amount in μM. Absorbance at 270nm.

Table 2. Malonaldehyde(MA) concentration in liver microsome depending on thawing numbers

No. of thawing	MA concentration (nmole/mg/min)	%
0	0.723± 0.007	100.0
1	0.518± 0.004	71.7
2	0.475± 0.002	65.7
3	0.383± 0.004	53.0
4	0.327± 0.004	45.2

Values represent mean± SD of five microsomal suspensions incubated in Fe^{+3}-ADP/NADPH lipid peroxidation system. Each value was significantly different from one another by Student's t-test, $P<0.005$.

Antioxidant activity of carotenoids and DL-α-tocopherol by TBA value

One of the most extensively employed methods for the detection and analysis of peroxidation is the TBA test, predicated upon the reactivity of a colorless aldehyde end-product of lipid peroxidation, malondialdehyde(MDA), with TBA to produce a red adduct. MDA is considered to be derived from lipid peroxides of polyunsaturated fatty acids with three or more double bonds[23]. In spite of nonspecificity of TBAs chemical reactivity and the low efficiency of fatty acid hydroperoxide breakdown to MDA, this method is still widely used because of its convenience, ease, and rapidity.

Carotenoids and DL-α-tocopheral showed antioxidant activities by inhibiting MDA formation induced by Fe^{+3}-ADP/NADPH and paraquat/NADPH system(Table 3). Their antioxidant activities showed similar trends in both systems ; lycopene, lutein and α-carotene had more inhibitory effect on lipid peroxidation than β-carotene or DL-α-tocopherol, known physiologic antioxidant at the concentration of 1×10^{-4}M. As shown in Fig. 1, the structural differences in carotenoids are cyclization, addition of hydroxyl group on β-ionone ring and cis-trans isomerization. From Table 3, structure-activity relationship can be proposed. 1) α-carotene showed more antioxidant activity than β-carotene ; cis configuration on β-io-

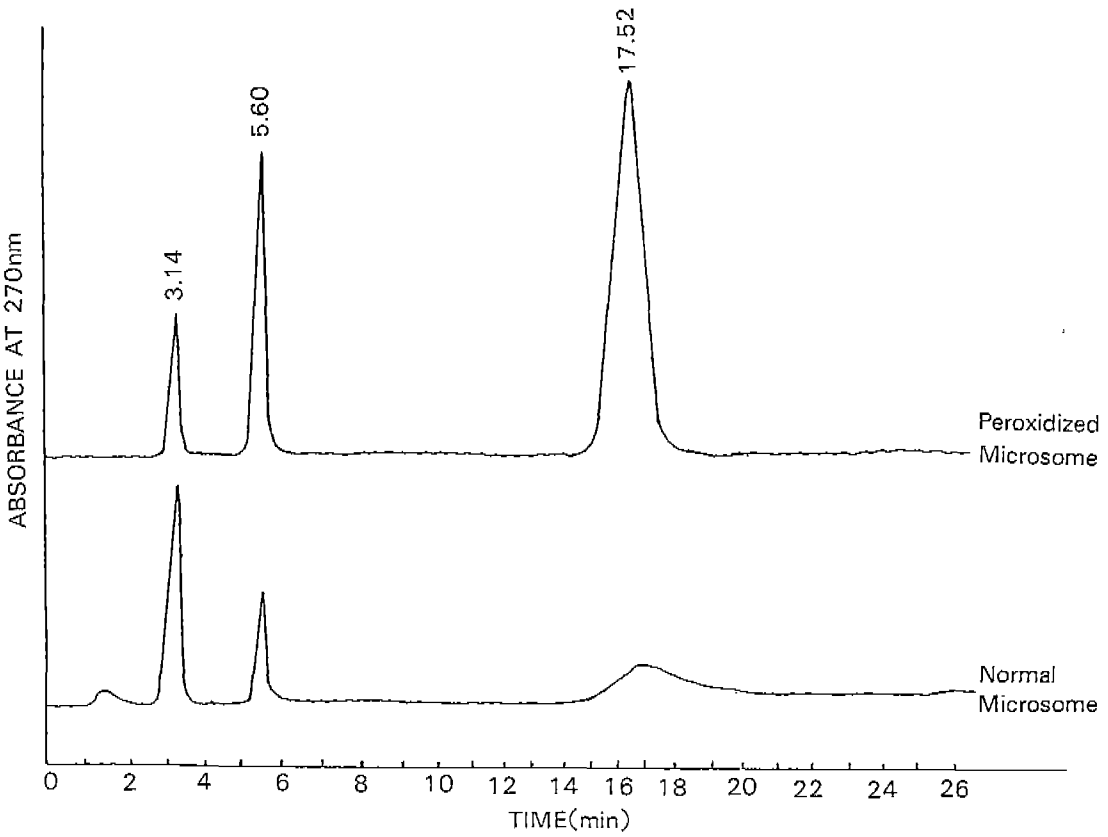

Fig. 4. HPLC separation of malonaldehyde in normal and peroxidized microsomal suspension (1mg protein/ml) incubated in Fe^{+3}-ADP/NADPH system at 37℃ for 30min. Retention times are given above the peaks in minutes ; nicotinamide(3.14), and malonaldehyde(5.60).

none ring may better trap radical species than trans form. 2) Lutein had similar antioxidant activity as α-carotene ; hydroxyl group at the 3 and 3'position on ionone ring does not affect the activity of α-carotene. 3) Uncyclization on the carotenoid structure shows efficient antioxidant activity. Second proposal about lutein and α-carotene is in agreement with very recent presentation by Park [24]. He found the β-carotene and zeaxanthin(β, β-carotene-3, 3'diol) possessed similar antioxidant activities. It was also suggested that canthaxanthin and astaxanthin which have oxo group seem to be more effective than β-carotene and zeaxanthin by stabilizing the trapped radicals[24].

Comparison of HPLC analysis with the TBA value

The concentrations of TBARS and MA in normal microsomes were almost same as 0.721 and 0.701 nmole/mg protein/min, respectively. Inhibitory effects of carotenoids and DL-α-tocopherol were also similar when measured by TBARS or by MA. This result demonstrated that lipid peroxidation system stimulated by Fe^{+3}-ADP/NADPH forms only free MA and no significant amounts of other MA-like substances. Therefore, TBA value seems a good indicator for lipid peroxidation in this particular system.

Antioxidant activities of carotenoids and DL-α-

Table 3. Effect of carotenoids and DL-α-tocopherol on Fe^{+3}-ADP/NADPH or Paraquat/NADPH induced lipid peroxidation by TBA method

Treatment	Fe^{+3}-ADP/NADPH System		Paraquat/NADPH System	
	MDA nmole/mg/min	% Control	MDA nmole/mg/min	% Control
None	0.740± 0.049	100	0.290+0.021	100
α-carotene	0.466± 0.048**	63	0.203± 0.019**	70
β-carotene	0.592± 0.052**	80	0.238± 0.016**	82
Lutein	0.496± 0.053**	67	0.194± 0.015**	67
Lycopene	0.429± 0.031**	58	0.194± 0.014**	67
DL-α-tocopherol	0.651± 0.040*	88	0.244± 0.020**	84

Rat liver microsomes(1mg protein/ml) were incubated with 100μM carotenoids or DL-α-tocopherol in lipid peroxidation systems in vitro. % control indicates percentage of MDA(malondialdehyde) produced by control with no treatment. Values represent the mean± SD of five determinations. Asterisks indicate values significantly different from none-treatment value by Student's t-test. *$p<0.01$, **$p<0.005$.

Table 4. Comparison of inhibitory effect of carotenoids and DL-α-tocopherol on Fe^{+3}-ADP/NADPH induced lipid peroxidation by TBARS and malonaldehyde contents.

Treatment	TBARS		Malonaldehyde	
	nmole/mg/min	% Control	nmole/mg/min	% Control
None	0.721± 0.059	100	0.701± 0.063	100
α-carotene	0.454± 0.062***	63	0.491± 0.054***	70
β-carotene	0.577± 0.053***	80	0.561± 0.048***	80
Lutein	0.483± 0.067***	67	0.484± 0.051***	69
Lycopene	0.418± 0.061***	58	0.435± 0.049***	62
DL-α-tocopherol	0.634± 0.048*	88	0.575± 0.059**	82

Rat liver microsomes were incubated with 100μM carotenoids or DL-α-tocopherol in Fe^{+3}-ADP/NADPH peroxidation system. TBA reactive substances(TBARS) were determined by colorimetric method and malonaldehyde was measured by HPLC. % control indicates percentage of control value with no addition. Values represent the mean± SD of five determinations. Asterisks indicate values significantly different from none-treatment value by Student's t-test, *$p<0.05$, **$p<0.01$, ***$p<0.005$.

tocopherol were compared by TBA value(contents of TBA reactive substances) and HPLC analsys (Malonaldehyde content in peroxidized liver microsomes). In Fe^{+3}-ADP/NADPH lipid peroxidation system, β-carotene and DL-α-tocopherol showed similar antioxidant activities. Lycopene had more efficient radical-quenching activity than lutein and α-carotene both by TBA reactive substances(TBARS) and by malonaldehyde(MA) analysis.

Conclusively, in present study the antioxidant activitives of carotenoids were compared with relation to their structures and their antioxidant activities were also compared with that of DL-α-tocopherol, natural antioxidant largely present in cellular membranes, by means of HPLC analysis(MA content) and TBA value. α-Tocopherol acts as an antioxidant by cleavage of the chroman ring system to a quinone[25]. The specific chemical reactions of carotenoids has not been clarified except that the polyene chain is broken when carotenoids interact with radical species[26]. Further researches should be concentrated on the mechanism of action of each carotenoid for its biological functions either antioxidant activity which contributes to anti-

cancer and antimutagenic effects or others.

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고압 액체 크로마토그라피에 의한 알파-, 베타- 카로텐, 루테인 및 리코펜의 항산화효과 비교 연구

김 혜 영

한국인삼연초 연구소 인삼효능 연구실

국문초록

과산화지질 반응 계에서 말론알데히드 정량을 위한 HPLC방법을 개발하였으며, 알파-, 베타- 카로텐, 루테인 및 리코펜의 간 마이크로좀 시스템에서의 항산화작용을 새로 개발한 HPLC 방법과 TBA 값으로 비교하였다. 해동횟수에 따른 간 마이크로좀의 말론알데히드 형성능력을 비교하였다. 결과로서, 말론알데히드는 5·6분만에 확인되었으며, pcak area와 농도는 직선 관계를 나타내었다. 해동횟수 증가에 따라 마이크로좀의 지질과산화 능력은 감소되었으며, 이는 세포막의 파괴에 기인한것으로 사료된다. Fe^{+3}-ADP/NADPH와 paraquat/NADPH 반응계에서 리코펜, 루테인 및 알파-카로텐은 베타-카로텐이나 토코페롤보다 강력한 항산화효과를 나타내었다. Fe^{+3}-ADP/NADPH 반응 계에서 카로테노이드와 토코페롤의 과산화지질 형성 억제효과는 TBA 값과 HPLC에 의한 말론알데히드 정량에 의해 비교해볼때 유사한 효과를 나타내었다.