RADICAL SCAVENGING ACTIVITY OF EXTRACTS OF ARGENTINE PEANUT SKINS (ARACHIS HYPOGAEA) IN RELATION TO ITS *TRANS*-RESVERATROL CONTENT

Nepote,V.[1]; Grosso, N.R.[1]; Guzman, C.A.[2]

[1] Química Biológica, FCA (UNC), IMBIV (CONICET), Avda. Valparaiso s/n, CC 509, 5000 Córdoba, Argentina.

Fax: +54 351 4334116/7/8, E-mail:nrgrosso@agro.uncor.edu

[2] Instituto de Ciencia y Tecnología de los Alimentos, FCEFyN (UNC), IMBIV (CONICET), Av. Velez Sarsfield 1600. 5000 Córdoba, Argentina.

Received February 10, 2004. In final form June 24, 2004.

Abstract

The objective of this work was to determine radical scavenging activity in relation to the content of total phenolic compounds and trans-resveratrol (resveratrol) in fractions from ethanolic extracts of peanut skins from Argentina. Ethanolic extract was prepared from defatted peanut skins. Total dry matter content, total phenolic content and radical scavenging activity percentage were determined from ethanolic extract and purified fractions. The ethanolic extract was separated by partition with dichloromethane, ethyl acetate and water. The ethyl acetate fraction was separated in a minicolumn packed with Sephadex LH-20 because this fraction showed higher content in phenolic compounds and radical scavenging activity. The fractions from the column were analyzed by paper chromatography and thin layer chromatography to identify resveratrol. HPLC and ^{1}H-NMR spectroscopy were performed in resveratrol fraction. Ethanol extracted 0.1056g of dry matter per gram of defatted peanut skins. Ethanolic extract showed 0.932g/g of total phenolic compound and 31.5% of radical scavenging activity. The resveratrol content was 91.4 ± 7.3μg/g in ethanolic extract and 9.07 ± 0.72μg/g in peanut skins.

Resumen

El objetivo de este trabajo fue determinar la actividad secuestrante de radicales libres en relación con el contenido de fenoles totales y trans-resveratrol en fracciones de extractos etanólicos obtenidos a partir de tegumento de maní argentino. El extracto etanólico se obtuvo a partir de tegumento de maní deslipidizado. Sobre el extracto etanólico crudo y las fracciones purificadas se determinaron los contenidos de materia seca total, fenoles totales y actividad secuestrante de radicales libres. El extracto etanólico fue separado por partición con diclorometano, acetato de etilo y agua. La fracción de acetato de etilo fue separada en una minicolumna empacada con Sephadex LH-20 debido a su mayor contenido en fenoles totales y actividad secuestrante de radicales libres. Las fracciones obtenidas de la columna se analizaron mediante cromatografía en capa delgada y papel para identificar el resveratrol. Sobre las fracciones con resveratrol se realizaron determinaciones por HPLC y ^{1}H-NMR. El

etanol extrajo 0.1056g de material seca por gramo de tegumento de maní deslipidizado. El extracto etanólico resultó con un contenido de 0.932g/g de fenoles totales y 31.5% de actividad secuestrante de radicales libres. El contenido de resveratrol fue de 91.4 ± 7.3μg/g en el extracto etanólico y 9.07 ± 0.72μg/g en el tegumento de maní.

Introduction

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a stilbene phytoalexin produced by different parts of plants like grapes and peanuts. Stilbenes are low-molecular-weight, biologically active metabolites produced by plants as a defense response to various exogenous stimuli, particularly, a fungal challenge [1-4].

Resveratrol has been shown to possess cancer chemo-preventive activity in mice and to act as an antioxidant and antimutagen [5]. It is also associated with reduced risk of cardiovascular disease by inhibiting or altering platelet aggregation and coagulation, or modulating lipoprotein metabolism [6-11]. It has been demonstrated that wine preference in moderated quantities was associated with a significantly lower risk for cardiovascular death, which possibly could be explained by higher consumption of wines containing resveratrol [12].

Earlier research on resveratrol in the 1970s and 1980s [1] indicated that the compound was only found in peanuts that had been inoculated and incubated with microorganisms, making them inedible. But more recent works [13] had found that resveratrol is present, even without any indication of fungal attack.

Resveratrol has been found in grapes, grape skins and grape products, such as wines at 0.031-7.17 ppm levels [14,15]. Sanders *et al.* (2000) [13] adapted analytical methods used for resveratrol in wine to isolate and identify resveratrol in peanuts. It was examined Spanish, Runner and Virginia-type peanuts from different production locations in the United States. In edible peanuts, resveratrol was found at levels of 0.02-1.79 $\mu g\ g^{-1}$ [13] and in peanut products around 0.018-7.873 $\mu g\ g^{-1}$ [4].

Peanut skins are a waste from blanched processing of peanut kernels. In Argentina, peanuts skins are used to feed cattle. However, their value could be increased if other more valuable uses were found. At present, studies of resveratrol were not undertaken in Argentina peanuts and peanut skins. The purpose of this work was to determine radical scavenging activity in relation to the content of total phenolic compounds and *trans*-resveratrol (resveratrol) content in fractions from ethanolic extracts of peanut skins from Argentina.

Materials and methods

Materials. Peanut skins from Argentine peanuts (cv Florunner, 2000 crop year) were obtained by a blanching process and were provided for the Company "Lorenzati, Ruesch y Cia", Ticino, Cordoba, Argentina in July, 2000. The peanut skins were kept in a sealed plastic bag and stored at 4°C until used.

Obtaining ethanolic extract (EE) from peanut skins. To obtain the EE, the peanut skins were previously defatted by two extractions with n-hexane (50mL each 10g peanut skins) during 12 hours each one at room temperature. The dry defatted peanut

skins (20g) were extracted with 300mL ethanol during 24 hours by maceration in darkness at room temperature. The extract was filtered and the residue was extracted again under the same conditions. The combined filtrate was evaporated to dryness in a rotary evaporator (Buchi R 110, Frawil, Switzerland) at 35°C. The yield of dry matter of EE was determined on dry peanut skins basis. All extractions were run by triplicate and averaged.

Separation of the ethanolic extract. The crude EE was purified by partition with 90 mL dichloromethane (DCM), 300 mL ethyl acetate (EtOAc) and 55 mL water. The EtOAc fraction was evaporated in a rotary evaporator and separated with methanol in a minicolumn packed with Sephadex LH-20 (internal diameter: 10mm, length: 33.5 cm, elution flow 0.5 ± 0.05mL/min). Fractions with different colors in UV light were separated from the column. The fractions were analyzed using paper chromatography, thin layer chromatography, high performance liquid chromatography and ^{1}H nuclear magnetic resonance.

Identification and quantitative estimation of resveratrol from the column fractions. Thin layer chromatography (TLC) and paper chromatography (PC).

TLC was carried out on plates of silica gel (20x20cm and 0.5mm thick of silica gel 60G, Merck, Darmstadt, Germany.) using EtOAc:Acetic acid:Water (17:1:2) as eluting solvent. Descending PC was carried out on Whatman 3MM chromatography papers (20x20cm) using Methanol:water (2:1) as eluting solvent [16]. The crude extract and the purified fraction were compared with a standard of *trans*-resveratrol (99%, Sigma Co, St Louis, MO, USA). The spots were observed in UV light (365nm), and compared with exposure at NH_3 vapor.

Nuclear magnetic resonance (NMR). The ^{1}H NMR was only performed in the fraction where resveratrol was detected by TLC and PC using a Bruker NMR spectrometer (Bruker AC-200, Germany) at 200.13 MHz, chemical shifts are given in ppm. The standard resveratrol and the purified sample were dissolved in deuterated methanol (MeOD), with tetramethylsilane (TMS) as internal standard.

High performance liquid chromatography (HPLC). The purified fractions containing resveratrol were mixed and evaporated under a nitrogen atmosphere, the residue was dissolved in a HPLC mobile phase and injected (10 µL) in a high performance liquid chromatograph (LKB Bromma, Sweden) equipped with silica gel columns (Macherey-Nagel Nucleosil 120-5-C18, Düren, Germany). Resveratrol was separated with methanol:water (1:1 v:v, flow 1.5mL/min) as a mobile phase and identified and quantified using an UV detector at 320nm by reference to the peak area of an external standard of trans-resveratrol (Sigma Co). The calibration curve of standard resveratrol was: peak area = 8839168.45 x resveratrol concentration (mg/mL) + 108988.73 (R^2 = 0.92).

Determination of total phenolic compounds. The total phenolic compounds present in the peanut skins ethanolic extract and purified fractions were determined spectrophotometrically using the Folin-Ciocalteu method according to Waterman and Mole (1994) [17] adapted to a micro scale. In a 10mL volumetric flask, 8.4mL of deionized water, 0.1mL of sample diluted (3mg of crude extracts and fractions in 10mL of ethanol) and

0.5mL Folin-Ciocalteu reagent (Anedra, San Fernando, Buenos Aires, Argentina) were added, and the content of the flask was mixed thoroughly. After 1 min, 1 mL of Na_2CO_3 solution (0.2g/mL) was added and finally made up to 10 mL with deionized water. After 1 hour, absorbance was measured with a spectrophotometer (Spectronic 21, Bausch and Lomb, USA) at 760nm. The concentration of total phenolic compounds in extracts of peanut skins was determined by comparison with the absorbance of standard phenol (Merck, Darmstadt, Germany) at different concentration. All tests were run in triplicate, analyses of all samples were run in duplicate and averaged.

Determination of radical-scavenging activity (%RSA). %RSA of the ethanolic extracts and purified fractions were determined using diphenyl picryl hydrazyl radical (DPPH) (Aldrich, Milwaukee, WI, USA) according to Joeux *et al.* (1995) [18,19]. 8, 37.5 and 75µL methanolic solution of peanut skin extract (300µg/mL) were added to a 1.5 mL methanolic solution of DPPH radical (20µg/mL) to get a final concentration of 1, 5 and 10µg/mL, respectively. The mixture was shaken vigorously and left to stand for 5min. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. The radical-scavenging activity percentage was calculated using the formula: %RSA = [1 – (absorbance of DPPH and sample – absorbance of sample) / absorbance of DPPH] x 100. All tests were run in triplicate, analyses of all samples were run in duplicate and averaged.

Statistical analysis. Statistical analysis were conducted with the InfoStat software package (InfoStat software, version 1.1, 2002, Facultad de Ciencias Agropecuarias, Universidad Nacional de Cordoba, Cordoba, Argentina) of replicate test data. Analysis of variance were performed by ANOVA procedures. Significant differences ($\alpha = 0.05$) between mean values were determined by Duncan tests.

Results and discussion

EE from peanut skins. The yield of extraction with ethanol from the defatted peanut skins was 0.1056 ± 0.0023g dry matter of ethanolic extract per gram of defatted peanut skins, and 0.0992 ± 0.0022g dry matter of ethanolic extract per gram of peanut skins.

Total phenolic compounds of EE was of 0.932 ± 0.033g per gram of ethanolic extract. Therefore, defatted peanut skins had 0.0984 ± 0.0021g phenolic compounds per gram. Duh *et al.* (1992) [20] reported 0.0199g phenolic compounds per gram of peanut hulls using ethanol as extraction solvent. This result is indicating that peanut skins could be a higher source of phenolic compounds and natural antioxidant components than peanut hulls.

RSA percentages in 1, 5 and 10µg/mL EE were 31.5 ± 0.9, 89.4 ± 4.1 and 97.1 ± 0.3, respectively. Yen and Duh (1994) [21] found radical-scavenging activity of 89.3% inhibition in 1500µg/mL methanolic extract from peanut hulls. EE from defatted peanut skins showed a higher radical-scavenging activity than the extracts from peanut hulls.

Separation of EE. Table 1 shows the yield of dry matter extraction, total phenolic content and RSA percentage from the partition of EE with DCM, EtOAc and water. The fraction of EtOAc showed significantly higher yield extraction (0.435g/g EE) and total

phenolic content (0.836g/g EtOAc) than the fractions of DCM and water (ANOVA and Duncan test, $\alpha = 0.05$). Due to these results, the EtOAc fraction was separated by using a chromatography column. The RSA percentages were higher in EtOAc (39.72%) and water (39.66%) fractions with no significant differences between them with respect to that of the DCM fraction (0.20).

Table 1*. Yield of dry matter, total phenolic content and radical scavenging activity of partition extracts from ethanolic extract: dichloromethane (DCM), ethyl acetate (EtOAc) and water.*

	Yield of Extraction [a]	**Total Phenolic Content** [b]	**% Radical Scavenging Activity** [c]
		(mean ± SE)[d]	
DCM	0.1998 ± 0.0066 C [e]	0.049 ± 0.002 C	0.20 ± 0.00 B
EtOAc	0.4350 ± 0.0118 A	0.836 ± 0.081 A	39.72 ± 0.21 A
H_2O	0.3265 ± 0.0098 B	0.668 ± 0.032 B	39.66 ± 1.16 A

[a] *Expressed as g of dry extract/g of dry ethanolic extract.*
[b] *Determined with the Folin-Ciocalteu method, expressed as g phenolic compound/g dry extract.*
[c] *Determined with 1μg extract/mL methanolic dilution and DPPH radical, expressed as percentages of Radical Scavenging Activity.*
[d] *Values are mean ± standard error of triplicate analyses.*
[e] *Mean values within a column with the same upper case letter are not significantly different at $\alpha = 0.05$.*

Table 2 presents UV colors and yield of dry matter extraction of 8 EtOAc fractions eluted from the column. The fraction III showed fluorescent blue color that might indicate the resveratrol presence [1]. This fraction had a yield of 0.2497g/g. The fractions V-VIII exhibited the same color at UV, (purple), indicating that their composition could be similar. These fractions had 0.5206g/g yield. Total phenolic compounds (Fig. 1) and radical-scavenging activity (Fig. 2) of 8 fractions eluted from column oscillated between 0.63g/g (V) - 1.09g/g (II) and 37.26% (fraction III) – 49.81% (fraction VI), respectively. No significant differences were found in the total phenolic compounds and RSA of the eight EtOAc fractions obtained from the column (ANOVA, $\alpha = 0.05$).

Identification of resveratrol by TLC, PC and NMR. The results of TLC and PC are presented in Table 3 and 4, respectively. Only resveratrol was identified among the spots. The examination of TLC and PC of resveratrol standard under UV light revealed the presence of a single fluorescent blue spot at R_F 0.91 in TLC, and 0.64 in PC. The spot color did not change with NH_3 vapor.

The resveratrol spot did not appear in EE and the fractions of EtOAc, water and DCM because of low concentration. The II and III column chromatography fractions showed one spot whose color and R_F were similar to standard resveratrol. Then the fraction II and

III were joined to be analyzed by NMR and HPLC. The NMR spectra of resveratrol standard and the sample (fraction II and III) showed a triplet at 6.26ppm and a doublet at 7.37ppm. Similar values were found for resveratrol by other authors [22, 23, 24]. The sample had different peaks between 6.3–7.2 ppm when compared with standard resveratrol indicating the presence of other phenolic compounds.

Table 2. *UV color, volume of methanol elutes, and yield of extraction of EtOAc fractions from column chromatography.*

CC Fraction	mL eluted methanol	UV Color	Yield of Extraction [a]
I	11.6	Green	0.0168 ± 0.0061
II	47.8	Yellow	0.0207 ± 0.0020
III	31.4	Fluorescent blue	0.2497 ± 0.018
IV	12.8	light blue	0.1923 ± 0.012
V	75.9	Purple	0.2747 ± 0.020
VI	104.5	Purple	0.1332 ± 0.0246
VII	151.9	Purple	0.0657 ± 0.0074
VIII	244.3	Purple	0.0470 ± 0.0093

[a] *Expressed as g of dry extract/g dry ethanolic extract. Values are mean ± standard error of triplicate analyses.*

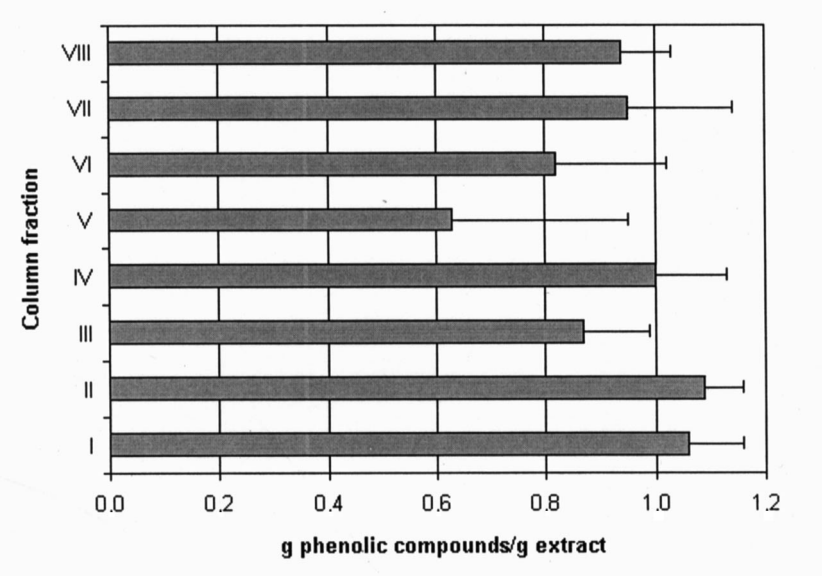

Figure 1. *Total phenolic content of EtOAc fractions from the column chromatography.*

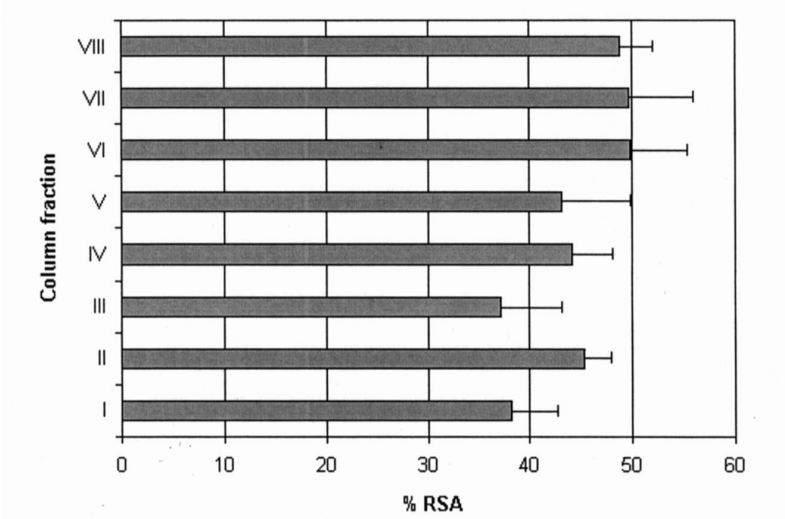

Figure 2. *Radical scavenging activity percentage (%RSA) of 1μg/mL EtOAc fractions from the column chromatography.*

Table 3. *Color and R_F of the spots separated by thin layer chromatography*

Rf	Spot color (UV) Fractions from the column chromatography								Standard
	I	II	III	IV	V	VI	VII	VIII	resveratrol
0.36	purple	purple							
0.47	purple	yellow	yellow						
0.53	Purple	purple							
0.61	Purple	purple							
0.63				purple	purple	Purple	purple		
0.67				purple	purple	Purple	purple		
0.79			purple	purple	purple	Purple	purple	purple	
0.89			purple	purple	purple	Purple	purple		
0.91		fluores-cent blue	fluores cent blue						fluores cent blue

Table 4. *Color and R_F of the spots separated by thin layer chromatography*

Rf	Spot color (UV) Fractions from the column chromatography								Standard
	I	II	III	IV	V	VI	VII	VIII	resveratrol
0.0						Purple	purple	purple	
0.2	Yellow	yellow	yellow	Yellow	Yellow	Yellow			
0.5		Fluores cent blue	light blue Fluores cent blue						fluores cent blue
0.6									
0.7			purple	Purple	Purple	Purple	purple	purple	
0.8			purple	Purple	Purple	Purple	purple	purple	

Quantitative estimation of resveratrol by HPLC. The HPLC analysis is presented in Fig. 3. the retention time of standard resveratrol was 3.77 min. In the sample, the resveratrol peak appeared at the same retention time.

The resveratrol content was of 91.4 ± 7.3µg/g dry EE, and 9.07± 0.72 µg per gram of dry peanut skins. In other works related to resveratrol in grapes and wine, this compound has been found in concentrations between 0.031-7.17 ppm [14, 15]. In edible peanuts from the United States, resveratrol was found at levels of 0.02-1.79µg/g [13] and in peanut products around 0.018-7.873 µg/g [4]. Comparing with those amounts, peanut skins from Argentina had higher content of resveratrol than peanuts and peanut products from the USA.

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are used in many food products to prevent rancidity in spite of growing concern for the potential health hazards of synthetic antioxidants. One example is the work from Ito *et al.* [25] that reported BHA to be carcinogenic in animal experiments. This work renews interest in the increased use of naturally occurring antioxidants in the food industry. Peanut skins could be a source of natural antioxidants as resveratrol, that implies benefits for the consumers health as antioxidant and in the prevention of cancer and cardiovascular diseases [5, 6, 7, 8, 9, 10, 11].

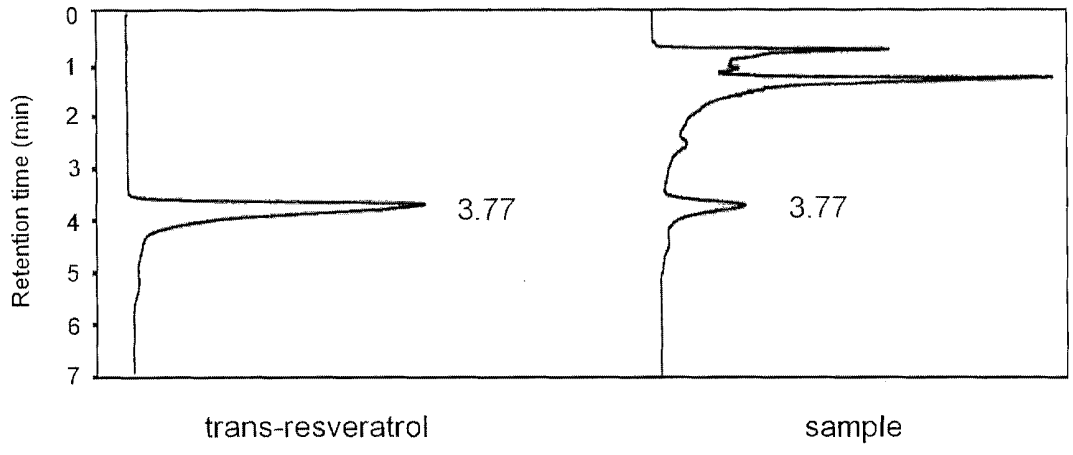

Figure 3. *High performance liquid chromatography analysis of resveratrol and sample (fractions II and III from the column).*

Acknowledgments

We thank CONICET and SECYT-UNC for financial support and the Lorenzati, Ruesch company for provision of peanut skins.

References

[1] Ingham, J. L. *Phytochemistry* **1976**, *15*, 1791.
[2] Adrian, M.; Jeandet, P.; Veneau, J.; Weston, L. A.; Bessis, R. *Journal of Chemical Ecology* **1997**, *23*, 1689.
[3] Sobolev, V. S.; Cole, R. J.; Dorner, J. W.; Yagen, B. *J AOAC Int.* **1995**, *78*, 1177.
[4] Sobolev, V. S.; Cole, R. J. *J Agric Food Chem.* **1999**, *47*, 1435.
[5] Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218.
[6] Arichi, H.; Kimura, Y.; Okuda, H.; Baba, K.; Kozawa, M.; Arichi, S. *Chem Pharm Bull.* **1982**, *30*, 1766.
[7] Kimura, Y.; Okuda, H.; Arichi, S. *Biochim Biophys Acta* **1985**, *834*, 275.
[8] Kimura, Y.; Okuda, H.; Kubo, M. *J Ethanopharmacol* **1995**, *45***,** 131.
[9] Chung, M-I; Teng, C-M; Cheng, K-L; Ko, F-N; Lin, C-N. *Planta Med.* **1992**, *58*, 274.
[10] Bertelli, A. A. E.; Giovannini, L.; Giannessi, D.; Migliori, M.; Bernini, W.; Fregoni, M.; Bertelli, A. *Int. J. Tiss. React.***1995**, *17*, 1.
[11] Pace-Asciak, C. R.; Hahn, S.; Diamandis, E. P.; Soleas, G.; Goldberg, D. M. *Clin. Chim Acta.* **1995**, *235***,** 207.
[12] Klatsky, A. L.; Armstrong, M. A.; Friedman, G. D. *Ann Intern Med.* **1992**, *117*, 646.
[13] Sanders, T. H.; McMichael, R. W. Jr.; Hendrix, K. W. *J Agric Food Chem.* **2000**, *48*, 1243.
[14] Mattivi, F. *Z Lebensm-Unters Forsch.* **1993**, *196*, 522.
[15] Celotti, E.; Ferrarini, R.; Zironi, R.; Conte, L. S. *J Chromatogr. A.* **1996**, *730,* 47.
[16] Wagner, H.; Bladt, S.; Zgainski, E. M. Plant Drug Analysis. A Thin Layer Chromatography Atlas. Springer-Verlag New York, USA. **1984**, *163-192*.
[17] Waterman, P. G.; Mole, S. Analysis of Phenolic Plant Metabolites. *Blackwell Scientific Publications,* Oxford, UK. **1994**.
[18] Joeux, M.; Lobstein, A.; Anton, R.; Mortier, F. *Planta Medica* **1995**, *61*, 126.
[19] Schmeda-Hirschmann, G.; Razmilic, I.; Gutiérrez, M. I.; Loyola, J. I. *Economic Botany* **1999**, *53*, 177.
[20] Duh, P.; Yeh, D.; Yen, G. *J. Amer. Oil. Chem. Soc.***1992**, *69*, 814.
[21] Yen, G.; Duh, P. *J. Agric. Food Chem.* **1994**, *42*, 629.
[22] Mannila, E.; Talvitie, A.; Kolehmainen, E. *Phytochemistry* **1993**, *33*, 813.
[23] Mattivi, F.; Reniero, F.; Korhammer, S. *J. Agric. Food Chem*.**1995**, *43*, 1820.
[24] Oleszek, W.; Sitek, M.; Stochmal, A.; Peacente S.; Pizza, C.; Cheeke, P. *J. Agric. Food Chem.* **2001**, *49*, 747.
[25] Ito, N.; Hagiwara, A.; Shibata, M.; Ogiso, T.; Fukushima, S. *Gann.* **1982**, *73*, 332-334.