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Development of a reverse-phase high-performance liquid chromatography method for analyzing *trans*-resveratrol in peanut kernels

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Abstract

A reverse-phase high-performance liquid chromatography (HPLC) method for identification and quantification of *trans*-resveratrol from peanut extracts, using phenolphthalein as internal standard, was developed. The HPLC system consisted of a C_{18} column (250 l × 4.6 i.d. mm, 5 μm particle size), with PDA detection at 307 nm and mobile phase consisting of 0.1% acetic acid in water and 100% acetonitrile. Gradient elution increased acetonitrile linearly from 5% to 41.8% over 23 min (GS = 1.6) followed by an increase of acetonitrile to 77% over 5 min (GS = 7.04), returning to initial conditions over 1 min and held for an additional 5 min with a flow rate of 1.5 ml/min. A lower column temperature resulted in higher peak heights and better baseline separation, therefore 25 °C was selected over 40 or 60 °C to simplify the method. Accuracy, precision, linearity, limit of detection and limit of quantitation were consistent or better than reported previously in the literature for related studies.

Keywords: Resveratrol; Peanuts; Reverse-phase; HPLC

1. Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is an antioxidant compound synthesized in plants. Identification and quantification of resveratrol in food has recently become the target of intense research due to health promoting activities (Frankel, Waterhouse, & Kinsella, 1993; Jang et al., 1997; Gehm, McAndrews, Chien, & Jameson, 1997; Docherty et al., 1999).

Resveratrol is most commonly identified in peanuts and related products by high-performance liquid chromatography (HPLC) analysis (Table 1). Quantification of resveratrol in peanut kernels stressed, by slicing (2 mm) and incubating at 25 °C for 24–48 h, by reverse-phase HPLC was first performed by Cooksey, Garratt, Richards, and Strange (1988). HPLC reverse-phase methods are typically more convenient and "robust" than other forms of liquid chromatography (Snyder, Kirkland, & Glajch, 1997). Normal-phase methods have also been used to quantify resveratrol in peanuts and wine (Table 1); however not as commonly as reverse-phase. Vinas, Lopez-Erroz, Marin-Hernandez, and Hernandez-Cordoba (2000) selected reverse-phase chromatography because polyphenols in wine are insoluble in water but soluble in alcohols and the stationary phase (Spherisorb ODS-2 column) permitted superior separation.

Resveratrol in peanuts by HPLC is commonly analyzed using gradient elution rather than isocratic methods (Table 1). The percent of mobile phase solvents

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Table 1
Comparison of published methods of HPLC analysis for resveratrol and other related phytoalexins in peanuts and grapes and their related products and standard solutions

Sample	Separation mode[a]	Column	Column temperature (°C)	Mobile phase solvents			Elution	Flow rate (ml/min)	Detector
				A	B	C			
Peanut kernels[b,c]	Reverse-phase	Hypersil Octadecylsilyl (ODS) (250 l × 4.6 i.d. mm; 5 μm particle)	NR	Acetonitrile/ water (1/1, v/v)	NA	NA	Isocratic	4	Pye Unicam LC-UV 335 nm Tekman potentiometric chart recorder
Peanut kernels[d]	Reverse-phase	Spherisorb 10 ODS (250 l × 4.6 i.d. mm; 5 μm particle)	NR	Water/acetic acid (9/1, v/v)	Acetonitrile	NA	Gradient: time (min), % B; 1, 40; 7, 45; 12, 45; 20, 65	1.5	Multichannel detector 310 nm and 0.04 A
Peanut kernels[e]	Reverse-phase	Spherisorb 10 ODS (250 l × 4.6 i.d. mm; 5 μm particle)	NR	Water/acetic acid (9/1, v/v)	Acetonitrile	NA	Gradient: time (min), %B; 1, 30; 3, 30; 6, 35; 9, 35; 12, 40; 15, 40;18, 50; 20, 50; 21, 30; 31, 30	1.5	Philips diode array (PDA) UVmode, 338 nm
Wine[f]	Reverse-phase	Nucleosil C_{18} 120 (250 l × 4 i.d. mm; 5 μm particle)	40	Water/acetic acid (pH 2.4)	Solvent A/acetonitrile (2/8 v/v)	NA	Gradient: time (min), %B; 0, 18; 10, 18; 17, 23;21, 24.5; 27, 31.5; 30, 100; 35, 18	1.5	PDA 306 and 285 nm
Wine[g]	Reverse-phase	ODS-II	NR	Water/acetonitrile/ acetic acid (70/29.9/0.1, v/v)	NA	NA	Isocratic	NR	UV detector 280 nm
Wine[h]	Reverse-phase	Nucleosil C_{18}[i] (150 l × 4.6 i.d. mm; 5 μm particle) Benzyldimethylsislyl bonded phase (150 l × 4.6 i.d. mm; 7 ± 2 μm particle)	NR	0.05 mol/l ammonium phosphate in 25% acetonitrile	NA	NA	Isocratic	NR	Variable-wavelength UV 200–800 nm Electrochemical glassy carbon electrode and silver/silver chloride reference electrode
Wine[j]	Normal-phase	Lichrosphere 100 CN (250 l × 4 i.d. mm; 5 μm particle)	NR	Water/ acetonitrile/ methanol (90/5/5, v/v)	NA	NA	Isocratic	1.0	Variable wave length UV 306 nm PDA 240–280 nm (*trans*- and *cis*-resveratrol at 306 nm)
Peanut kernals[k]	Normal-phase	Ultrasphere-SI (250 l × 4.6 i.d. mm; 5 μm particle)	NR	*n*-Heptane/ 2-propanol/water/ acetonitrile/acetic acid (1050/270/17/5/1, v/v)	NA	NA	Isocratic	1.5	Programmable multiwavelength 300 nm or 290–345 nm range (12 fixed wavelengths; 5 nm increments) in 0.005–0.005 AUFS range

Wine[l]	Reverse-phase	ODS hypersil (250 l × 4 i.d.mm;, 5 μm particle) guard LiChrosphere 100 C_{18} (4 l × 4 i.d. mm; 5 μm particle)	NR	Acetic acidz	Methanol	Water	Gradient: time (min), %A/B/C; 0, 5/15/80; 5, 5/20/75; 30, 5/45/50; 40, 5/45/50; 50, 5/15/80;	0.4, 0.5, 0.5, 0.5, 0.4	PDA 266, 280, 306, 317, 369 nm (*trans*- and *cis*-resveratrol at 306 nm)
Standard solution[m]	Reverse-phase	ODS Hypersil (100 l × 2.1 i.d. mm; 5 μm particle)	40	Water	Methanol	NA	Gradient: time (min), %B; 0, 0; 15, 100; 16, 0; 21, 0	0.5	PDA 306 and 286 nm (*trans*- and *cis*-resveratrol at 306 and 286 nm)
Grapevine leaves[n]	Reverse-phase	Lichrocart Merck C_{18} (250 l × 4 i.d. mm; 5 μm particle)	NR	Water	Acetonitrile	NA	Gradient: time (min), %B; 0, 10; 18, 85; 23, 85; 30, 10; 35, 10	1.0	PDA 250–400 nm (*trans*-resveratrol at 307 nm) Fluorometer 330/374 nm (excitation/emission)
Roasted and boiled peanuts and peanut butter[o]	Normal-phase	Zorbax-RX-SIL (250 l × 4.6 i.d. mm; 5 μm particle)	Ambient	*n*-Hexane/ 2-propanol/ water/acetonitrile/ acetic acid (1050/270/17/5/1, v/v)	NA	NA	Isocratic	1.5	PDA detector 220–450 nm (*trans*-resveratrol at 307 nm for fresh and roasted peanuts and 320 nm for peanut butter)
Grape Juice[p]	Reverse-phase	Tracer Nucleosil C_{18} 120 (250 l × 4 i.d. mm; 5 μm particle)	40	Water/acetic acid (900/52.6, v/v)	Solvent A/ acetonitrile (20/80, v/v)	NA	Gradient: time (min), %B; 0, 16.5; 13, 18; 15, 18; 17, 23; 21, 25; 27, 31.5; 30, 0	1.5	PDA 306 and 285 nm (*trans*- and *cis*-resveratrol at 306 and 285 nm)
Peanut kernels[q]	Reverse-phase	Vydac C_{18} (150 l × 4.5 i.d. mm; 5 μm particle)	NR	Water/TFA (9.9/0.1, v/v)	Acetonitrile	NA	Gradient: time (min), %B; 1, 0; 3, 15; 23, 27; 28, 100; 29, 0; 39, 0	NR	UV 308 nm
Peanut butter[r]	Reverse-phase	Nucleosil 120 C_{18} (250 l × 4 i.d. mm; 5 μm particle)	40	Acetic acid/water (52.6/900, v/v)	Solvent A/ acetonitrile (2/8, v/v)	NA	Gradient time (min), %B; 0, 16.5; 13, 18; 15, 18; 17, 23; 21 25; 27, 31.5; 30, 0	1.5	PDA detector 285 and 306 nm (*trans*- and *cis*-resveratrol at 306 and 285 nm)
Wine[s]	Reverse-phase	Spherisorb ODS-2 (150 l × 4 i.d. mm 5 μm particle)	Ambient	Acetic acid/water (5/95, v/v)	Acetonirrile	NA	Gradient: time (min), %B; 0, 9; 10, 9; 11, 25; 22, 25; 23, 70; 28, 70; 29, 9; 44, 9	1.0	PDA 280, 360 and 300 nm (*trans*- and *cis*-resveratrol at 300 nm) Fluorescence detector 280/315, 324/370, 260/370 (excitation/emission; *trans*- and *cis*-resveratrol at 324/370 and 260/370 nm)
Grapes[t]	Reverse-phase	Hypersil ODS (150 l × 4.6 i.d. mm; 5 μm particle)	40	Acetonitrile	Water/acetic acid/acetonitrile (87/3/10, v/v)	NA	Gradient: time (min), %B; 0, 95; 25, 75	1.0	PDA 190–390 nm (*trans*- and *cis*-resveratrol at 306 nm)

(*continued on next page*)

Table 1 (*continued*)

Sample	Separation mode[a]	Column	Column temperature (°C)	Mobile phase solvents			Elution	Flow rate (ml/min)	Detector
				A	B	C			
Wine[u]	Reverse-phase	Symmetry C_{18} (150 l × 2.1 i.d. mm; 5 μm particle)	NR	Water/sulfuric acid (pH 2.5)	Acetonitrile	NA	Gradient: time (min), %B; 0, 0; 60, 50; 90, 50; 100, 100; 110, 0	0.2	PDA 240–305 nm (*trans*- and *cis*-resveratrol at 305 and 280 nm) Mass spectrometer selected ion monitoring at *m/z* 228
Wine[v]	Reverse-phase	Nova-Pak C_{18} (150 l × 3.9 i.d. mm; 4 μm particle)	NR	Methanol/acetic acid/water (10/2/88, v/v)	Methanol/acetic acid/water (90/2/8, v/v)	NA	Gradient: time (min), %B; 0, 0; 15, 15; 25, 50; 34, 70	1.0	PDA 280 nm Fluorescence detector 278/360 nm over 17.5 min, 330/374 nm for 16.5 min (excitation/emission)
Grape skin[w]	Reverse-phase	LiChrosphere 100 C_{18} (125 l × 4 i.d. mm; 5 μm particle) Lichrospher 100 C_8 (125 l × 4 i.d. mm; 5 μm particle)	NR	Acetic acid/ Methanol/water (5/20/75, v/v)	NA	NA	Isocratic	0.5, 1.0, 1.5 or 2.0	UV–Vis detector 306 nm
Wine[x]	Reverse-phase	Nova-Pak (150 l × 3.9 i.d. mm; 5 μm particle)	NR	Methanol/acetic acid/water (10/2/88, v/v)	Methanol/acetic acid/water (90/2/8, v/v)	NA	Gradient: time (min), %B; 0, 0; 10, 0; 25, 15; 35, 50; 44, 70	1.0	UV–Vis detector 280 nm PDA wavelengths were NR
Grape and cranberry juice, and wine[y]	Reverse-phase	Hypersil ODS (100 l × 2.1 i.d. mm; 5 μm particle)	25	Formic acid/ water (0.5/99.5, v/v)	Methanol	NA	Gradient: time (min), %B; 0, 25; 28, 39; 29, 95; 34, 95	0.25, 0.25, 0.5 and 0.5	Mass spectrometer positive ion atmospheric pressure; chemical ionization; selected ion monitoring at *m/z* 229
Peanut roots[z]	Reverse-phase	Thermal Hypersil ODS (250 l × 4 i.d. mm; 5 μm particle)	NR	Water	Methanol	NA	Gradient: time (min), %B; 1, 20; 16, 80; 18, 80	1.0	UV 254 nm

NA is not applicable.

NR is not reported information in the literature.

[a] Separation modes were conducted as normal or reverse-phase. Normal-phase HPLC utilizes a polar adsorbent as the stationary phase (column), such as silica or silica to which non-ionic functional groups have been chemically attached, and nonpolar mobile phase. Reverse-phase utilizes a nonpolar stationary phase and polar mobile phase. The stationary phase in reverse-phase systems are chemically bonded phases of slica surface silanols with an organochlorosilane. Usually the R_3 group is a octadecyl (C_{18} chain) as in octadecylsilyl (ODS) bonded phases (Rounds and Gregory, 1998).

[b] Aguamah et al. (1981).

[c] Three phytoalexins, 4-(3-methyl-but-l-enyl)-3,5,3′,4′-tetrahydroxy-stilbene, 4-(3-methyl-but-2-enyl)-3,5,4′-trihydroxystilbene (4-isopentenylresveratrol) and 4-(3-methyl-but-l-enyl)-3,5,4′-trihydroxystilbene, closely related to resveratrol were isolated from peanuts.

[d] Cooksey et al. (1988).

[e] Arora and Strange (1991).

[f] Lamuela-Raventos et al. (1995)).

[g] Pezet et al. (1994).

[h] McMurtrey et al. (1994).

[i] Nucleosil C_{18} column was used for all quantitative determinations.

and the gradient steepness (GS), % the less polar solvent increase over time in reverse phase systems (Snyder et al., 1997), varies greatly between analysis methods (Table 1). Phenolic compounds, like resveratrol, are highly soluble in common reverse-phase HPLC mobile phase solvents, such as methanol/water (Trela & Waterhouse, 1996) and acetonitrile/water (Siemann & Creasy, 1992) systems. Vinas et al. (2000) found that methanol in the mobile phase led to problems of high pressure and consequently chose acetonitrile instead. A dilute acid, such as acetic acid, in water is also commonly incorporated as one of the mobile phase solvents in reverse-phase systems (Table 1). Addition of acid in the mobile phase improves analysis by suppressing on-column ionic dissociation of the three phenolic hydroxyl groups, acidic, of resveratrol (Wang, Catana, Yang, Roderick, & Van Breemen, 2002).

Using elevated column temperatures above ambient, 25 °C (Sobolev & Cole, 1999) during HPLC analysis of resveratrol has not been commonly practiced. Several studies used thermostatically controlled heating to maintain a column temperature of 40 °C in order to identify resveratrol (Table 1). Currently there are no studies in the literature that compare the effect of column temperature on HPLC analysis of resveratrol.

Detection and quantification of *trans*-resveratrol by different researchers was by UV absorption at 306–308 nm wavelengths for HPLC analysis (Table 1). Trela and Waterhouse (1996) determined the molar absorptivities of *trans*- and *cis*-resveratrol in 100% ethanol at UV λ_{max} 308 and 288 nm, respectively, and 306 and 286 nm, respectively, in 10% ethanol using a diode array spectrophotometer. The slight difference in absorptivity between the two solutions was attributed to altered hydrogen bond interactions with the presence of water (Trela & Waterhouse, 1996). Consistent results were found for *trans*-resveratrol in 100% methanol (Jeandet et al., 1997) and *n*-hexane–2-propanol–water–acetonitrile–acetic acid (1050/270/17/5/1, v/v) solution (Sobolev & Cole, 1999).

The majority of published studies (Table 1) have used PDA detectors because they allow simultaneous detection of multiple wavelengths therefore allowing detection a range of compounds (Soleas et al., 1997) and prevent improper quantitation of compounds by checking peak purity and spectral identity (Snyder et al., 1997). Other less common methods used for detection of resveratrol in grapes and grape products were HPLC with fluorometric detection (Jeandet et al., 1997; Rodriguez-Delgado, Malovana, Perez, Borges, & Garcia Montelongo, 2001) or electrochemical detection (McMurtrey, Minn, Pobanz, & Schultz, 1994), gas chromatography–mass spectrometry (GC–MS) analysis (Goldberg et al., 1994), liquid chromatography-mass

[j] Goldberg et al. (1995).
[k] Sobolev et al. (1995).
[l] Goldberg et al. (1996).
[m] Trela and Waterhouse (1996).
[n] Jeandet et al. (1997).
[o] Sobolev and Cole (1999).
[p] Romero-Perez et al. (1999).
[q] Sanders et al. (2000).
[r] Ibern-Gomez et al. (2000).
[s] Vinas et al. (2000).
[t] Palomino et al. (2000).
[u] Dominguez et al. (2001).
[v] Rodriguez-Delgado et al. (2001).
[w] Pascual-Marti et al. (2001).
[x] Malovana et al. (2001).
[y] Wang et al. (2002).
[z] Chen et al. (2002).

spectrometry (LC-MS) analysis (Dominguez, Guillen, & Barroso, 2001; Wang et al., 2002) and capillary electrophoresis with UV detection (Arce, Tena, Rios, & Valcarcel, 1998; Berzas Nevado, Contento Salcedo, & Castaneda Penalvo, 1999). Although some of these other detection methods are at least as good as or better than PDA they are seldom used for resveratrol analysis because of additional sample preparation or availability of equipment.

Internal standards, such as 3,4,5-trimethoxycinnamic acid (Dominguez et al., 2001) and 2,5-dihydroxybenzaldehyde (Malovana, Garcia Montelongo, Perez, & Rodriguez-Delgado, 2001), were used in reverse-phase HPLC methods with PDA detection for analysis of *trans*-resveratrol in wine. Although some researchers have developed reverse-phase HPLC methods with gradient elutions and PDA detection (Table 1) for determining resveratrol in peanuts and peanut products, use of an internal standard has not been investigated. However, Sobolev, Cole, and Dorner (1995) included phenolphthalein as an internal standard to help quantify stilbenes, including *trans*-resveratrol, in peanut samples analyzed by normal-phase HPLC. Sobolev et al. (1995) concluded that phenolphthalein was a stable internal standard with a suitable retention time relative to the stilbenes analyzed and helped to quantitatively determine the stilbenes in the sample. Hence it is of interest to be able to accurately determine resveratrol in peanuts and peanut products using an internal standard in a reverse-phase HPLC system.

The main objective of this study was to develop a reverse-phase HPLC method using PDA detection for the quantification of *trans*-resveratrol in peanuts using phenolphthalein as the internal standard. Specific objectives were to: (1) determine a reverse-phase HPLC mobile phase gradient solvent composition range for elution of *trans*-resveratrol and phenolphthalein, (2) identify a GS that effectively separates *trans*-resveratrol from other eluting compounds in peanut extracts using PDA, (3) investigate the effect of column temperature on *trans*-resveratrol elution, (4) validate the mobile phase gradient solvent composition, GS and column temperature by testing for accuracy, precision, linearity, limit of detection (LOD) and limit of quantitation (LOQ), and (5) quantitatively determine resveratrol in the peanuts using phenolphthalein as an internal standard.

2. Materials and methods

2.1. Reagents and standards

Ethanol (AAPER Alcohol, Shelbyville, KY), acetonitrile (Aldrich Chemical Company, Milwaukee, WI) and glacial acetic acid (J.T. Baker, Phillipsburg, NJ), HPLC grade, were used. Water used was double deionized and filtered, by vacuum, through a 0.2 μm nylon filter (Millipore Corporation, Bedford, MA). Standard stock solutions for phenolphthalein (Aldrich Chemical Company, Milwaukee, WI) and *trans*-resveratrol (Sigma-Aldrich, St. Louis, MO), each containing 200 ppm (200 μg/ml), were prepared separately by quantitatively transferring approximately 0.01 g of powder to a 50 ml volumetric flask, and bringing to volume with 100% ethanol. Prior to storage of *trans*-resveratrol standard the flask was flushed with nitrogen gas (medical grade, Air Products and Chemicals, Inc., Allentown, PA) to remove oxygen and wrapped with aluminum foil to reduce light-induced isomerization (Trela & Waterhouse, 1996). Stock solutions were stored at −5 °C for up to 3 mo (Trela & Waterhouse, 1996). Working solutions for both compounds were prepared daily to contain 20 ppm by dilution of stock solutions (200 ppm) with 10% ethanol in a 10 ml volumetric flask. Preparation of *trans*-resveratrol solutions was conducted under yellow light to prevent isomerization (Trela & Waterhouse, 1996).

2.2. Materials

Peanuts used in the analysis were Georgia green medium runners (McCleskey Mills Inc.) harvested in Smithville, GA in 2001. The 50 Ib bag of peanuts had been stored under refrigerated storage in a cold room at 7 °C for approximately 9 mo prior to analysis. Grinding of peanut samples for extraction was done using a coffee mill (Model K9M2-4, BrAun, Mexico). Peanuts and extraction solvent were homogenized (PowerGen 700,

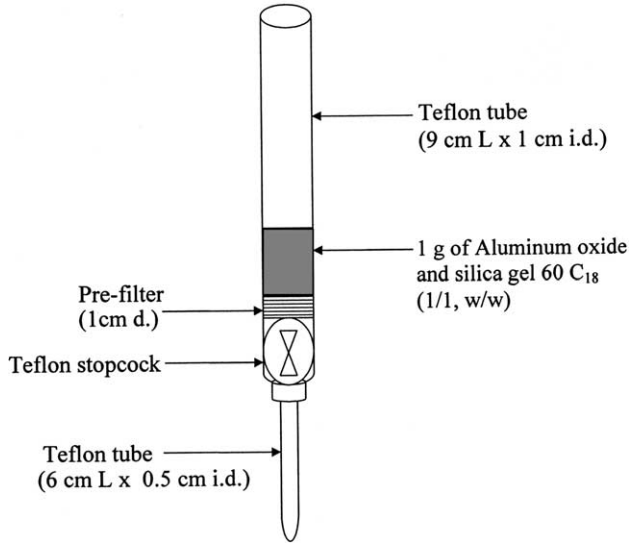

Fig. 1. Schematic diagram of clean-up column used for peanut sample purification.

Fisher Scientific, Pittsburgh, PA). Samples were centrifuged (Model J2-21M, Beckman, Palo Alto, CA) at 1380 G using # 14 rotor.

A clean-up column, used for sample purification, was constructed by fitting a series of Teflon tubes (Nalgene, Rochester, NY). See Fig. 1 for schematic diagram of clean-up column. Prior to attaching the top Teflon tube to the stopcock a pre-filter (AP25, Millipore, Bedford, MA), was precut to fit using a cork borer (Boekel Scientific, Feasterville, PA) from 12 mm to 1 cm in diameter, and placed at the bottom of the column to prevent loss of packing. The clean-up column was packed with 1 g of 1/1 (w/w) mixture of aluminum oxide (neutral, activity 1, particle size 0.063–0.200 mm; EM Science, Gibbstown, NJ) and silica gel 60 C_{18} (EM Science, Gibbstown, NJ).

Five ml of 80% ethanol was added to the column and the contents were stirred with a glass stirring rod to distribute the packing mixture equally throughout the column. Once the ethanol drained through the column, air bubbles were removed from the packing by pressing with a stirring rod and additional ethanol (ca. 2 ml), as needed. Clean-up columns were used once for each sample; the packing was discarded and Teflon parts were cleaned with detergent (Micro-90, International Products Corporation, Burlington, NJ).

Peanut extracts were dried with nitrogen gas blown directly over the contents of the vials, placed in a vial rack that was half submerged in water, maintained at 60 °C, in a glass Pyrex dish (29.21 L × 19.05 W cm) on top of a hot plate (Thermix®, model 2 10T, Fisher Scientific, Pittsburgh, PA). Peanut extracts were filtered with an inorganic membrane filter (Anotop 10, 0.2 μm, Whatman Internationl Ltd, Maidstone, England) attached to a glass syringe prior to HPLC analysis.

2.3. Peanut sample preparation

Trans-resveratrol was extracted from peanuts following a procedure established by Sanders, McMichael, and Hendrix (2000). Ten grams of ground peanuts, 2 ml of phenolphthalein solution (10 μg/ml) added as the internal standard and 30 ml of 80% ethanol were added to a 250 ml centrifuge tube (Nalgene, Rochester, NY). The contents of the tube were homogenized on setting 5, approximately 27,000 rpm, for 2 min on ice then centrifuged for 5 min at 1380 G. Two milliliter of the clear supernatant from the centrifuged sample was transferred, by pipette (5 ml, Pipetteman, Rainin Instrument Co., Woburn, MA), to a clean-up column in order to remove interfering compounds that co-elute with resveratrol (Sanders et al., 2000). The sample was allowed to drain by gravity through the column and was collected in a 4 ml vial (National Scientific Company, Lawrenceville, GA). After the sample completely drained, approximately 15 min, 2 ml of 80% ethanol was pipetted onto the column and collected in the same vial. The contents of the vial was evaporated to dryness under nitrogen gas, as described in Section 2.2. Samples that were not analyzed immediately were stored, in the dry state, at −5 °C in the vial, which was recapped and wrapped with aluminum foil to prevent isomerization.

Preparation of the sample for HPLC analysis involved dissolving the dried residue with 0.40 ml of 10% ethanol. In order to expose ethanol to all surfaces of the vial the lid was replaced and the entire vial was rotated, by hand, for 30 s then the vials were held halfway submerged under water in an ultrasonic bath and rotated for 10–15 s. Extracts were prepared for HPLC analysis by filtering the entire vial contents through a syringe filter (Lamuela-Raventos, Romero-Perez, Waterhouse, & de la Torre-Boronat, 1995) into a polypropylene plastic insert (300 μl, National Scientific Co., Lawrenceville, GA) which was placed in a 2 ml amber vial (National Scientific Co., Lawrenceville, GA). A screw cap and teflon/silicone septum (National Scientific Co., Lawerenceville, GA) were used to seal the vial. The entire extraction procedure was conducted under yellow light to prevent isomerization (Trela & Waterhouse, 1996).

Phenolphthalein was used as internal standard to calculate the amount of *trans*-resveratrol in the sample using a formula described by Macrae (1982). *Trans*-resveratrol and phenolphthalein standard concentrations and peak areas used in the equation were averages of 7 standards. Standards for *trans*-resveratrol and phenolphthalein, analyzed at the beginning of each HPLC sample set, contained a known concentration of each compound. Solutions used for generating standard curves and development of the HPLC method were prepared by taking 5 ml of *trans*-resveratrol (20 ppm) and 5 ml of phenolphthalein (20 ppm) working solutions for a total of 10 ml (10 ppm of each compound) in a volumetric flask. All subsequent standard solutions were made by taking 9.40, 7.55, 6.62, 4.89, 6.96, 6.25 or 3.30 ml from the higher solution in the series and diluting it with 10% ethanol in a 10 ml volumetric flask, producing 7 levels of standards at 9.4, 7.1, 4.7, 2.3, 1.6, 1.0, and 0.33 ppm, respectively. All solutions were prepared for HPLC analysis as described above. Peak areas for standards and samples were determined by HPLC analysis, method described in the next section.

2.4. HPLC analysis

Analysis was performed using a Waters (Waters Corporation, Milford, MA) HPLC system comprised of a Waters 717 sample injector, Waters 2695 separations module, and Waters 996 PDA set to monitor the UV

spectrum from 240 to 400 nm. The analytical column was heated using a column heater module (Waters Corporation, Milford, MA) equipped with a temperature control module (Waters Corporation, Milford, MA). The Waters Millenium[32] software, version 3.05 (Waters Corporation, Milford, MA) was used to control the HPLC auto sampler, gradient settings, PDA and data acquisition. *Trans*-resveratrol and phenolphthalein peak areas were determined at 307 (Sobolev & Cole, 1999) and 254 nm (Kowalczyki, Hawes, & McKay, 2000), respectively.

A C_{18} reverse-phase column, 250 l × 4.6 i.d. mm, 5 μm particle size (Econosphere, Alltech Associates, Inc., Deerfield, IL), was used. Preceding the analytical column was a C_{18} guard column, 7.5 l × 4.6 i.d. mm, 5 μm particle size (Econosphere, Alltech Associates, Inc., Deerfield, IL).

2.4.1. Development of reverse-phase gradient

The mobile phase consisted of 0.1% acetic acid in filtered deionized water as solvent A and 100% acetonitrile as solvent B. To determine the gradient conditions the method of Snyder et al. (1997) was used.

2.4.1.1. Determination of solvent composition. Initial and final composition of A and B solvents were determined using exploratory gradient elutions in two experiments. In experiment 1 acetonitrile was increased linearly from 5% to 100% over 60 min (1.6%/min, GS) with a flow rate of 1.5 ml/min (Cooksey et al., 1988; Arora & Strange, 1991; Sobolev et al., 1995; Sobolev & Cole, 1999; Ibern-Gomez, Roig-Perez, Lamuela-Raventos, & de la Torre-Boronat, 2000) and column temperature maintained at 40 °C. A solution of *trans*-resveratrol and phenolphthalein at 10 ppm each, was injected at 15 μl. In addition an experimental peanut extract containing natural concentration of *trans*-resveratrol and phenolphthalein at 2 ppm were injected at 40 μl. All analysis were conducted in triplicate. In order to improve sample analysis the retention times (T_r) of the first and last peaks observed in the chromatogram from the peanut samples were used to calculate the acetonitrile initial and final concentrations using a tabular value published by Snyder et al. (1997).

In experiment 2 the same conditions were used except for the initial and final gradient composition of acetonitrile which were adjusted to reflect the concentrations determined in experiment 1. The gradient composition was checked to ensure all peaks eluted in the new range and was adjusted to run over a 60 min period.

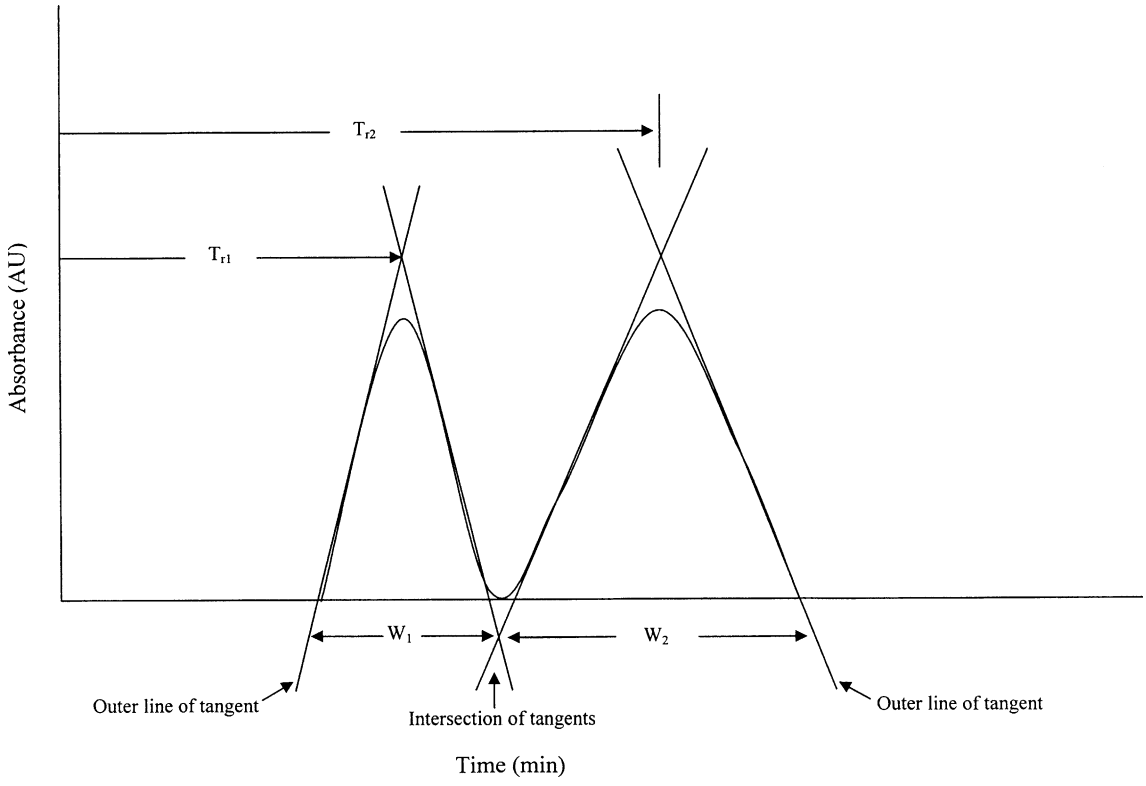

Fig. 2. Measurement of retention times (T_r) and baseline bandwidths (W) used for the calculation of resolution (R_s) of a HPLC analysis. Where $R_s = [2(T_{r2} - T_{r1})]/(W_1 + W_2)$; T_{r1} and T_{r2} = retention times of the first and second adjacent peaks and W_1 and W_2 = baseline bandwidths of the of first and second adjacent peaks measured between the intersection of the tangent to the outer tangent line (Snyder et al., 1997).

2.4.1.2. Determination of effect of gradient steepness and column temperature on resolution. The following set of experiments used the same conditions as experiment 2 except the gradient composition range was adjusted to occur within 45 min. This step was repeated using 30 min followed by 15 min to obtain 3 additional GS, to compare to GS at 60 min.

Each of these experiments was conducted at two column temperatures of 40 °C followed by 60 °C in succeeding experiments in order to determine if column temperature affected *trans*-resveratrol analysis. *Trans*-resveratrol and phenolphthalein in solution and peanut extract, described above, were analyzed at each of the 4 gradient steepness and 2 column temperatures, in triplicate, producing a total of 48 analyses. The General Linear Model (PROC GLM) and Fisher's least significant difference (LSD) were used to detect significant differences in retention times and peak heights of *trans*-resveratrol and phenolphthalein solution and peanut extract using Statistical Analysis Software (Version 8, SAS Institute Inc., Cary, NC).

Resulting chromatograms were used to calculate the resolution (R_s) of *trans*-resveratrol and phenolphthalein at each temperature and time where retention times of the first (T_{r1}) and second (T_{r2}) adjacent peaks and baseline bandwidths of first (W_1) and second (W_2) adjacent peaks were used in the equation described by Snyder et al. (1997). Determination of baseline bandwidth involves construction of tangents to each side of adjacent peaks then the distance between the intersection and outer tangent is measured (in mm) from each peak (Fig. 2). An R_s above 0.7 indicates that the method provides adequate separation (Snyder et al., 1997). The time and column temperature conditions that produced an R_s above 0.7 in the least amount of time and produced narrow peaks, determined by comparing peak height, was selected. This gradient steepness was used to determine the time period needed to allow both *trans*-resveratrol and phenolphthalein peaks to elute.

Recent studies (Table 1) have used ambient (25 °C) column temperatures when analyzing *trans*-resveratrol in peanuts (Sobolev & Cole, 1999) and wine (Vinas et al., 2000). To determine if ambient temperatures would yield similar peak heights and better baseline separation column temperatures of 25, 40 and 60 °C were used to analyze *trans*-resveratrol and phenolphthalein in solution and peanut extract.

2.4.1.3. Improvement by segmentation of the gradient elution. The gradient was adjusted to include a second segment with a steeper increase to the final concentration of acetonitrile in order to shorten analysis time. The second segment of the gradient elution was adjusted to occur within 5, 10 or 15 min while analyzing *trans*-resveratrol and phenolphthalein solution and peanut extract. To ensure that all compounds were eluted solvent was run through the system for, approximately 10 min, after acetonitrile reached the final concentration.

2.5. HPLC method validation

After the reverse-phase HPLC gradient conditions were determined validation tests were performed for accuracy, precision, linearity, range and limit of detection and quantitation.

2.5.1. Determination of accuracy

Determination of accuracy of the new HPLC method was done by recovery, where peanut extracts containing 0.5, 1.0, 1.5 ppm (μg/g peanut) of *trans*-resveratrol by adding 0.80, 1.6 and 2.4 ml, respectively, of the *trans*-resveratrol working solution (20 ppm) were compared with peanut extract without added standard. *Trans*-resveratrol concentration in all samples, determined by HPLC analysis, was calculated using the equation previously described in Section 2.3. Three replications were performed for each level of added *trans*-resveratrol resulting in 12 (4 concentrations × 3 replications) analysis. The % recovery was calculated from mean concentrations of three replications.

2.5.2. Determination of precision

To evaluate precision, one *trans*-resveratrol and phenolphthalein solution, prepared by taking 2.5 ml of each of the *trans*-resveratrol and phenolphthalein working solutions (20 ppm) and bringing it up to 10 ml with 10% ethanol in a volumetric flask, was injected at 15 μl for a total of 10 times. The precision of the method was expressed by the standard deviation (SD) and relative standard deviations (RSD) or coefficient of variation of the data set. The mean concentrations of *trans*-resveratrol and phenolphthalein were calculated. SD and RSD were calculated using equations described by Snyder et al. (1997). For analysis of compounds with low-level concentrations (ppm and ppb) precision of 1–2% RSD is acceptable (Snyder et al., 1997).

2.5.3. Determination of linearity

Linearity of the method was evaluated by injecting 15 μl of the 7 levels of *trans*-resveratrol and phenolphthalein standards, prepared as described in Section 2.3. Standard sets of 7 were analyzed in triplicate. The range of the seven standards used was established through consideration of published (Sobolev & Cole, 1999; Sanders et al., 2000; Ibern-Gomez et al., 2000), values of *trans*-resveratrol present in peanuts,

0.05–5.138 μg/g. Calibration curves for *trans*-resveratrol and phenolphthalein were constructed separately by plotting peak areas (*y*-axis) versus concentrations (*x*-axis) of the seven standards. Using Statistical Analysis Software (Version 8, SAS Institute Inc., Cary, NC) regression analysis (PROC REG) was used to relate *trans*-resveratrol and phenolphthalein standard concentrations individually as the dependent variable with peak area as the independent variable.

Pearsons product correlation (PROC CORR) coefficients (*r*) between peak area and the concentration of *trans*-resveratrol or phenolphthalein was analyzed using Statistical Analysis Software (Version 8, SAS Institute Inc., Cary, NC) for each of the seven standards used. A correlation coefficient above 0.999 is acceptable for most methods (Snyder et al., 1997).

2.5.4. Determination of LOD and LOQ

The LOD and LOQ were determined by analyzing *trans*-resveratrol and phenolphthalein solutions that were sequentially diluted in a series with 10% ethanol to obtain the lowest level of analyte that gave a measurable response with a signal-to-noise ratio of 3 and 10, respectively (Snyder et al., 1997). Signal is the height (mm) of the peak and noise refers to the amplitude or height (mm) of the baseline deflection. *Trans*-resveratrol and phenolphthalein solutions were prepared from working standards (20 ppm) by taking 0.5 ml and diluting with 10% ethanol to 10 ml in a volumetric flask resulting in a concentration of 1.0 μg/ml each. Subsequent solutions were made by taking approximately 5 ml of the previous solution and diluting it with 10% ethanol in a 10 ml volumetric flask. A 300 μl aliquot of each solution was placed into an insert which was inside of an amber vial and sealed with a screw cap and Teflon/silicone septum for HPLC analysis. Each solution was injected (15 μl) into the HPLC and the resulting chromatogram was used to measure the signal-to-noise ratio. Analyses were conducted in duplicate for each solution.

3. Results and discussion

3.1. Determination of solvent composition

The T_r of the first and last peaks observed in the chromatogram, from extracted peanut samples analyzed in experiment 1, were approximately 2.5 and 39 min, respectively (Fig. 3(a)). Using T_r of the first and last peaks and the table published by Snyder et al. (1997) the initial and final composition of acetonitrile to water was determined to be <1% and 77%, respectively. Results are similar to other gradients used in the literature for analysis of *trans*-resveratrol in solution (Trela & Waterhouse, 1996), peanuts (Sanders et al., 2000) and wine (Dominguez et al., 2001) where initial concentration of acetonitrile was 0%, however final concentrations of these studies were increased to 100%. Other researchers have used a final concentration of acetonitrile that is similar to 77% for analysis of grapes (Palomino, Gomez-Serranillos, Slowing, Carretero, & Villar, 2000) ending at 75% and wine (Vinas et al., 2000) ending at 70%.

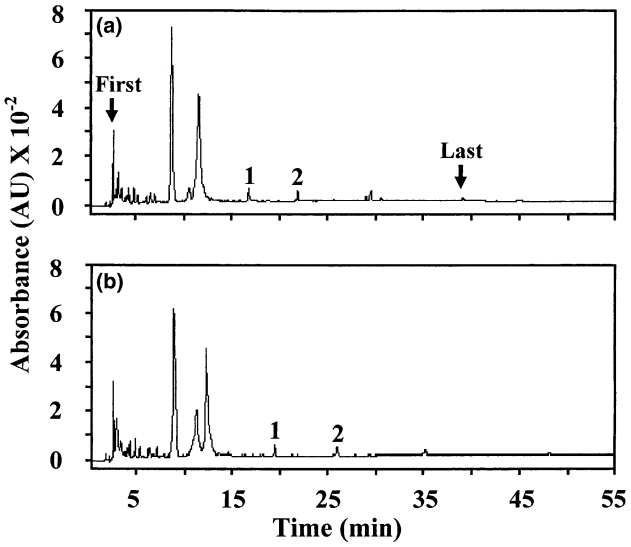

Fig. 3. Determination of a gradient solvent composition for reverse-phase HPLC analysis of *trans*-resveratrol and phenolphthalein in peanut extracts using photodiode array detection at 307 and 254 nm, respectively, with a flow rate of 1.5 ml/min, column temperature at 40 °C and analysis time of 60 min. (a) Retention time (T_r) of the first and last peaks, (**1**) *trans*-resveratrol and (**2**) phenolphthalein are shown in the chromatogram from analysis with a linear gradient increasing acetonitrile in acetic acid/water (0.1/9.9, v/v) from 5% to 100%. (b) The T_r of (**1**) *trans*-resveratrol and (**2**) phenolphthalein from analysis with a linear gradient increasing acetonitrile in acetic acid/water (0.1/99.9, v/v) from 5% to 77%.

Researchers have found that mobile phases that consist of 100% water can decrease the life of the analytical column. Snyder et al. (1997) suggests that the initial composition of water should start at 95% or lower in order to minimize exposure of the column to high water conditions. Therefore, new solvent initial and final concentrations were determined to be 5% and 77% acetonitrile, respectively. This new solvent system should prolong the life of the column.

The new solvent composition provided elution of all compounds in the solution and peanut extract as determined in experiment 2. However T_r was longer for both compounds (Fig. 3(b)) when compared to experiment 1 (Fig. 3(a)). The T_r of *trans*-resveratrol and phenolphthalein increased because the composition at which they elute occurred at a later time in the gradient of experiment 2.

3.2. Effect of gradient steepness and column temperature

The means of T_r and peak height of *trans*-resveratrol and phenolphthalein in solution and peanut extract resulting from analyses using a GS of 1.2, 1.6, 2.4 or 4.8 at column temperatures of 40 or 60 °C are presented in Table 2. Column temperature did not significantly ($p > 0.05$) affect retention time. These results are not in agreement with Snyder et al. (1997) who stated that increasing column temperature by 1 °C will usually slightly decrease T_r by 1–2%. As column temperature increased there was a significant ($p < 0.001$) decrease in peak height of both compounds in solution and peanut extract. Since narrow peaks, indicated by larger peak heights and short separation time are desirable in HPLC methods (Snyder et al., 1997) the analysis of *trans*-resveratrol and phenolphthalein in peanut extracts should be conducted using a maximum column temperature of 40 °C. Increased baseline separation for *trans*-resveratrol and phenolphthalein was also observed when column temperature was decreased from 60 to 40 °C (Fig. 4).

Decrease in GS significantly increased ($\alpha = 0.05$) retention time, as shown in Table 2, for *trans*-resveratrol and phenolphthalein in solution and peanut extract. As GS decreased peak height of *trans*-resveratrol and phenolphthalein in solution and peanut extract decreased significantly ($\alpha = 0.05$). The peak height was largest for *trans*-resveratrol and phenolphthalein in solution and peanut extract when analyzed in 15 min (GS = 4.8) at either column temperature (Table 2). Therefore analysis of *trans*-resveratrol should be conducted using the shortest analysis time (<60 min) and largest GS (>1.2) possible, which would result in good baseline separation.

Analysis within 15 min with a GS of 4.8 (Fig. 4(a) and (b)) and 30 min with a GS of 2.4 (Fig. 4(b) and (c)) producing the highest peak heights but resulted in poor baseline separation of *trans*-resveratrol from other compounds in the peanut extract. Analysis time at 45 and 60 min or GS of 1.6 and 1.2, respectively (Fig. 4(e)–(h)) resulted in increased baseline separation for *trans*-resveratrol, phenolphthalein and other interfering peaks in the sample.

The R_s of *trans*-resveratrol and phenolphthalein slightly increased when the column temperature was decreased from 60 to 40 °C (Table 3). A large increase was observed in R_s for *trans*-resveratrol and phenolphthalein as time increased from 15 to 60 min or GS of 4.8–1.2, regardless of column temperature (Table 3). Separation of *trans*-resveratrol with R_s above 0.7 occurred in samples analyzed for 45 or longer, whereas for phenolphthalein in peanut extracts a R_s above 0.7 was observed when analysis time was 15 min or longer (Table 3).

Table 2
Means of retention times (T_r) and peak heights of frvms-resveratrol and phenolphthalein solution and peanut extract analyzed by reverse-phase high performance liquid chromatography (HPLC) with photodiode array detection at 307 and 254 nm, respectively, using four analysis times with corresponding gradient steepness (GS) and 2 column temperatures[a,b]

Column temperature (°C)	Analysis time (min)	GS	T_r (min)				Peakheight (AU)[c]			
			Trans-resveratrol		Phenolphthalein		*Trans*-resveratrol		Phenolphthalein	
			10 ppm solution	Peanut extract	10 ppm solution	Peanut extract	10 ppm solution	Peanut extract	10 ppm solution	Peanut extract
40	15	4.8	8.88d	8.89d	10.69d	10.7d	6051a	9785a	4896a	6725a
	30	2.4	12.80c	12.81c	16.26c	16.26c	4527b	6223b	3734b	4232b
	45	1.6	16.18b	16.17b	21.25b	21.15b	3782c	5161c	3474c	3528c
	60	1.2	19.43a	19.37a	26.03a	25.97a	3102d	5087d	3287d	3244d
60	15	4.8	8.63d	8.59d	10.42d	10.42d	5322a	7689a	4299a	5752a
	30	2.4	12.34c	12.28c	15.81c	15.76c	3873b	4151b	3543b	3684b
	45	1.6	15.39b	15.39b	20.41b	20.41b	3238c	4138c	3366c	3366c
	60	1.2	18.97a	18.98a	25.25a	25.26a	3021d	4052d	3198d	3087d

[a] Mean ($n = 3$) attribute ratings in a column for each column temperature not followed by the same letter are significantly different ($\alpha = 0.05$) as determined by Fisher's least significant difference (LSD) mean separation test.

[b] *Trans*-resveratrol and phenolphthalein solutions and peanut extracts were injected at 0.015 and 0.040 ml, respectively into a gradient of acetonitrile in acetic acid/water (0.1/9.9, v/v) increasing from 5% to 77% with a flow rate of 1.5 ml/min. GS was (77–5%)/(analysis time).

[c] Analysis of variance of peak heights of frara-resveratrol and phenolphthalein, in solution and peanut extract, increased ($p < 0.0001$) as column temperature decreased from 60 to 40 °C.

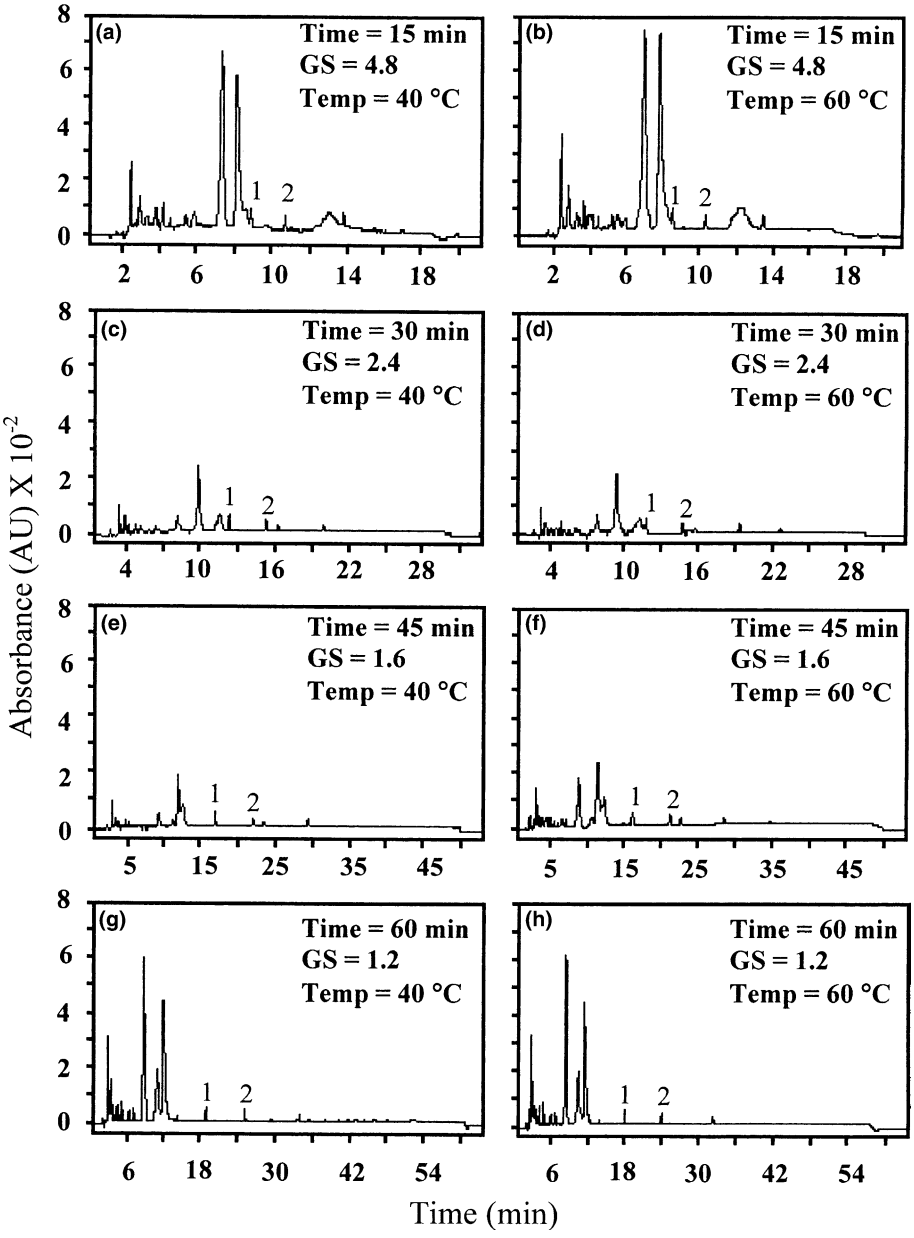

Fig. 4. Effect of reverse-phase HPLC gradient steepness (GS), of 1.2, 1.6, 2.4 and 4.8, and column temperature, at 40 and 60 °C, on analysis of *trans*-resveratrol and phenolphthalein in peanut extracts using photodiode array detection at 307 and 254 nm, respectively. Elution of (**1**) *trans*-resveratrol and (**2**) phenolphthalein from analysis with a linear gradient increasing acetonitrile in acetic acid/water (0.1/9.9, v/v) from 5% to 77% with varying analysis times, GS and column temperatures.

A new analysis time and GS of 45 min and 1.6, respectively, were the conditions that eluted *trans*-resveratrol and phenolphthalein with proper baseline separation and R_s above 0.7 in the shortest time. Using these conditions the T_r of *trans*-resveratrol and phenolphthalein in peanut extract were 16.17 and 21.15 min, respectively. Therefore, a time period of 23 min allowed both *trans*-resveratrol and phenolphthalein peaks to elute. No difference in peak height or retention time was observed when column temperature was maintained at 25 (ambient), 40 and 60 °C (Fig. 5). Based on the findings and to simplify analysis a column temperature maintained at 25 °C is recommended for analysis of peanut extracts.

3.3. Improvement by segmentation of the gradient elution

Improvement of analysis time was accomplished by using the GS of 1.6 for 23 min. An additional 5, 10 or 15 min allowed elution of all compounds (data not shown), however 5 min was selected to shorten total

Table 3
Resolution (R_s) of *trans*-resveratrol and phenolphthalein peaks from peanut extracts analyzed by reverse-phase HPLC with photodiode array detection at 307 and 254 nm, respectively, using four analysis times with corresponding gradient steepness (GS) and two column temperatures[a,b]

Analysis time (min)	GS	Column temperature (°C)	R_s	
			Trans-resveratrol	Phenolphthalein
15	4.8	40	0.16	1.82
15	4.8	60	0.15	1.81
30	2.4	40	0.33	2.01
30	2.4	60	0.27	2.00
45	1.6	40	1.80	2.20
45	1.6	60	1.77	2.10
60	1.2	40	2.30	2.90
60	1.2	60	2.10	2.80

[a] R_s = [2(retention time of second adjacent peak − retention time of first adjacent peak)]/(sum of baseline bandwidths of first and second adjacent peaks).
[b] *Trans*-resveratrol and phenolphthalein solutions (5 ppm) were injected at 0.015 ml into a gradient of acetonitrile in acetic acid/water (0.1/9.9, v/v) increasing from 5% to 77% with a flow rate of 1.5 ml/min. GS was (77–5%)/(analysis time).

analysis time. Therefore the second segment of the gradient was conducted in 5 min with a GS of 7.04.

The final gradient elution was determined to be the following: initial concentrations were 95% A and 5% B which were increased linearly to 58.2% A and 41.8% B over 23 min followed by a linear increase to 23% A and 77% B over 5 min and finally returned back to initial conditions, 95% A and 5% B, over 1 min and held for 5 additional min, to recondition the column. The flow rate and column temperature used in the elution are 1.5 ml/min and 25 °C, respectively.

3.4. HPLC method validation

3.4.1. Accuracy

Recovery of peanut extracts containing added *trans*-resveratrol at 0.5, 1.0, 1.5 ppm was 0.40 ± 0.02, 1.01 ± 0.01 and 1.44 ± 0.04 ppm corresponding to a percent recovery of 71.20 ± 3.42%, 96.08 ± 1.00% and 93.02 ± 2.35%, respectively. All calculations were adjusted to account for the natural concentration of *trans*-resveratrol, 0.50 ± 0.03 ppm (dry wt), determined in peanuts not containing added *trans*-resveratrol solution. Sobolev et al. (1995) found similar recovery of 96.05 ± 2.8% after adding 0.3 ppm of *trans*-resveratrol to peanut kernels ($n = 5$). Recoveries from the current study are higher than those found by Ibern-Gomez et al. (2000) where percentages were 81.07 ± 2.3, 75.37 ± 0.46 and 72.31 ± 3.86 for three concentrations, not specifically reported by the author, of *trans*-resveratrol added to peanut butter ($n = 3$ for each level). In a more recent study, Sobolev and Cole (1999) found recoveries of 117.23 ± 8.87%, 100.10 ± 2.49% and 100.45 ± 1.51% ($n = 3$) for 0.5, 5.0, and 10.0 ppm, respectively, when resveratrol standard was add to fresh peanuts which contained a natural level of 0.07 μg/g peanut. Natural concentration of *trans*-resveratrol in peanuts found in this paper are larger than reported by Sobolev and Cole (1999) at 0.07 μg/g but consistent with findings by Sanders et al. (2000) at 0.02–1.79 μg/g for peanuts in storage for up to 3 years.

3.4.2. Precision

The SD for *trans*-resveratrol was 0.0009 and RSD was 1.0126% (Table 4). Whereas, the SD of phenolphthalein

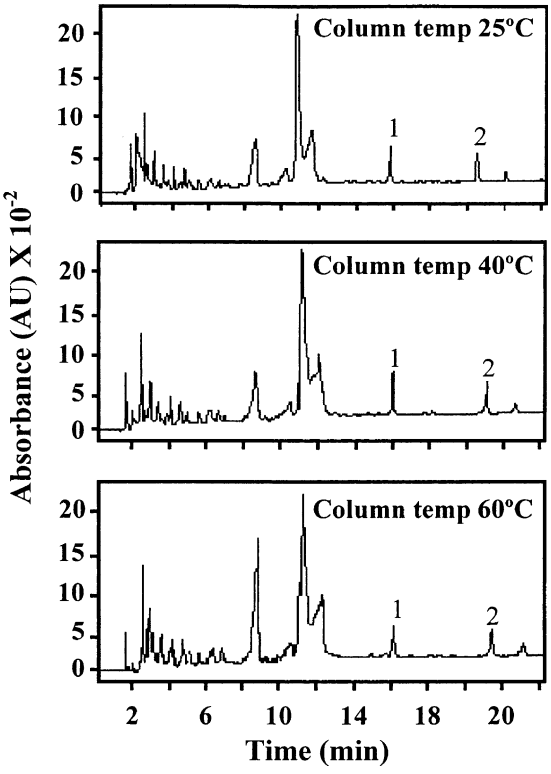

Fig. 5. Reverse-phase HPLC analysis of peanut extracts for the time period when (**1**) *trans*-resveratrol and (**2**) phenolphthalein eluted using photodiode array detection at 307 and 254 nm, respectively. The gradient linearly increased acetonitrile in acetic acid/water (0.1/9.9, v/v) from 5% to 41.8% over 23 min with a flow rate of 1.5 ml/min and varying column temperatures.

Table 4
Standard deviation (SD) and relative standard deviation (RSD) or coefficient of variation of *trans*-resveratrol and phenolphthalein concentration analyzed by reverse-phase HPLC using photodiode array detection at 307 and 254 nm, respectively[a,b]

Injection #	*Trans*-resveratrol[c]		Phenolphthalein[d]	
	Peak area (AU)	Concentration (ppm)	Peak area (AU)	Concentration (ppm)
1	335759	5.61	29147	4.95
2	334947	5.59	28331	4.81
3	333455	5.57	28574	4.85
4	333201	5.56	28552	4.85
5	331666	5.54	28500	4.84
6	330060	5.51	28508	4.84
7	329222	5.5	28471	4.83
8	327969	5.48	28452	4.83
9	326819	5.46	28415	4.82
10	326074	5.45	28414	4.82
SD		*0.0009*		*0.0006*
RSD		*1.0126*		*0.7893*

[a] RSD was calculated using the following equation: RSD(%) = (100 × SD)/mean of the ten readings.
[b] *Trans*-resveratrol and phenolphthalein solutions (5 ppm) were injected at 0.015 ml into a gradient of actetonitrile in acetic acid/water (0.1/9.9, v/v) increasing from 5% to 41.8% over 23 min then increasing to 77% over 5 min and finally returned to 5% over 1 min and held for an additional 5 min with a flow rate of 1.5 ml/min and column temperature at 25 °C.
[c] *Trans*-resveratrol concentrations using the regression equation and amount injected, $x = [(y + 675.94\ \mu g/AU)/4{,}000{,}000\ AU]/0.015$ ml where y is peak area and x is *trans*-resveratrol (ppm), are reported above.
[d] Phenolphthalein concentration using the regression equation and amount injected, $x = [(y - 505.85\ \mu g/AU)/385{,}681\ AU]/0.015$ ml where y is peak area and x is phenolphthalein (ppm), are reported above.

was 0.0006 and RSD was 0.7893% (Table 4). Results are better than acceptable limits, 1–2% (Snyder et al., 1997), for compounds of low-level concentrations. The RSD found in this paper was lower than others reported in the literature, 4.34% in peanut butter (Ibern-Gomez et al., 2000) and 1.56% in *trans*-resveratrol standard solutions (Lamuela-Raventos et al., 1995), using HPLC analysis. In addition our HPLC method provided lower SD and RSD than GC analysis of silyl derivatives of wine (Antonelli, Fabbri, & Lercker, 1996) where RSD, expressed as coefficient of variation by the author, was 4.88% and 3.85% for *trans*- and *cis*-resveratrol, respectively, with a SD of 0.02 for both isomers.

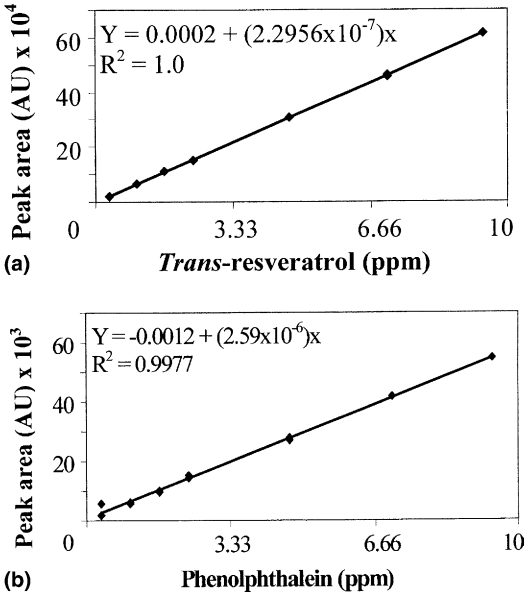

Fig. 6. Plots of peak area versus (a) *trans*-resveratrol and (b) phenolphthalein concentration determined by reverse-phase HPLC using photodiode array detection at 307 and 254 nm, respectively, and a gradient of actetonitrile in acetic acid/water (0.1/9.9, v/v) increasing from 5% to 41.8% over 23 min then increasing to 77% over 5 min and finally returned to 5% over 1 min and held for an additional 5 min with a flow rate of 1.5 ml/min and column temperature at 25 °C. Regression equations, where y is concentration and x is peak area, and coefficients of determination (R^2) are reported for each curve.

3.4.3. Linearity

Results of the regression analysis are presented in Fig. 6(a) and (b). Regression equations for both *trans*-resveratrol and phenolphthalein, having a coefficient of determination (R^2) of 1.0 and 0.9976, respectively, could be used to predict concentration from peak area.

Pearsons product correlation coefficient, r, for peak area and concentration of *trans*-resveratrol and phenolphthalein were 0.9999 and 0.9989, respectively, which are within the acceptable limits of $r > 0.999$ (Snyder et al., 1997). A recent study also found similar results (Ibern-Gomez et al., 2000) with $r \geqslant 0.999$ for *trans*-resveratrol standards, concentrations tested were not reported.

3.4.4. Limit of detection and limit of qualification

The LOD was determined to be 10 ppb (0.010 ppm) and 50 ppb (0.050 ppm) for *trans*-resveratrol and phenolphthalein, respectively. Results are lower than those found by Jeandet et al. (1997) using flourometric detec-

tion, where LOD at approximately 0.1 ppm fresh weigh grape leaves for four stilbenes, *cis*- and *trans*-resveratrol and its β-D-glucoside, ε-viniferin, and pterostilbene. In addition, results are better than Lamuela-Raventos et al. (1995) where LOD was determined at 3 ppm for measuring *trans*-resveratrol in wine.

LOQ was determined to be 0.04 and 1 ppm for *trans*-resveratrol and phenolphthalein, respectively. The LOQ for *trans*-resveratrol determined by our method is well below levels reported in the literature by Sobolev and Cole (1999) for detection in fresh peanuts and Lamuela-Raventos et al. (1995) in wine at 10 ppm.

4. Conclusion

A reverse-phase HPLC method for identification and quantification of *trans*-resveratrol from extracted peanut samples was developed in this study. The gradient and column temperature verified in this paper can only be successfully applied to a reverse-phase HPLC system with a C_{18} column (250 l × 4.6 i.d. mm, 5 μm particle size) maintained at 25 °C, with PDA detection and mobile phase consisting of 0.1% acetic acid in water and 100% acetonitrile. Improved analysis of *trans*-resveratrol in solution and peanut extract can be conducted with an HPLC reverse-phase gradient elution increasing linearly from 5% to 41.8% of acetonitrile over 23 min (GS = 1.6) followed by an increase of acetonitrile to 77% over 5 min (GS = 7.04), returning to initial conditions over 1 min and held for an additional 5 min with a flow rate of 1.5 ml/min. Results from the validation tests are consistent or better than reported previously in the literature for related studies. *Trans*-resveratrol was quantitatively determined using phenolphthalein as an internal standard in peanut extracts using the HPLC reverse-phase gradient elution and column temperature established in this study.

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