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Comparison of Different Extraction Solvent Mixtures for Characterization of Phenolic Compounds in Strawberries

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ABSTRACT: Eight different solvent mixtures containing acetone or methanol pure or combined with an acid (acetic, formic, hydrochloric) were tested for their efficiency for extraction of phenolic compounds from strawberries belonging to five groups of polyphenols: anthocyanins, flavonols, flavan-3-ols, hydroxycinnamic acid derivatives and conjugated forms of ellagic acid. Twenty-eight compounds from these five groups have been detected and quantified using HPLC−DAD−ESI-MS^n. The yield of each phenolic compound and group was evaluated with regard to the extraction solvent composition. Acetone containing extraction mixtures were superior to the ones containing methanol for extraction yield of total phenolic compounds, which was especially pronounced for the groups of flavan-3-ols and conjugated forms of ellagic acid. The mixture acetone/acetic acid (99:1, v/v) gave the best results for the qualitative and quantitative assay of the polyphenols present in strawberries since all 28 compounds were detected only in these extracts in quantities higher or comparable to the other extraction solvents tested.

KEYWORDS: extraction, acetone, methanol, strawberry, phenolic compounds, HPLC−DAD−ESI-MS

INTRODUCTION

The antioxidant potential of phytochemicals in health maintenance has been increasingly recognized in recent years. Sufficient evidence has shown that free radicals play an important role in most major health problems such as cancer, cardiovascular disease, and degenerative diseases associated with aging. Polyphenols are especially important antioxidants because of their high redox potentials allowing them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.[1] Plant phenols can be roughly divided in phenolic acids and flavonoids (flavanones, flavones, flavonols, anthocyanins, flavan-3-ols and their polymeric forms proanthocyanidins). They occur as free compounds, bound to sugars as glycosides, and can be further acylated with organic acids. Furthermore, tannins are more complex polyphenols occurring as hydrolyzable tannins (yielding gallic or ellagic acid when hydrolyzed) and condensed tannins, composed of flavonoid units joined by carbon−carbon bonds, which are not hydrolyzable, such as proanthocyanidins.

Berry fruits have been proven as rich sources of different polyphenols studied for their beneficial health effects as well as for their chemical characterization.[1−9] Strawberries are a very rich source of polyphenols in the human diet containing polyphenols from all classes. The variety and high content makes strawberries a very interesting sample for studies of polyphenols.[4,10−13] The critical point in studying polyphenols in plant materials is the extraction procedure used since it dictates the nature and quantity of polyphenols that will be transferred to the extract and further characterized. Different extraction procedures for studying phenolics in plant materials have been thoroughly reviewed by Naczk and Shahidi.[14] Methanol acidified with hydrochloric acid has been used for extraction of flavonoids and phenolic acids[6] and flavonols[7] in berries as well as for ellagic acid tannins and quercetin in raspberries,[15] and for anthocyanins and gallic acid derivatives from *Arbutus unedo* L. fruits.[16] Methanol−water−acetic acid mixture has been used for extraction and systematic study of anthocyanins in fruits and berries.[8,17] On the other hand, acetone−water mixture (70/30, v/v) has been used for survey of ellagitannins in berries,[18] and acetone/water/acetic acid mixtures for extraction of proanthocyanidins and anthocyanins in berries from the *Vaccinium* spp.,[9] for a study of proanthocyanidin oligomers and other phenolics in strawberry cultivars[12] as well as for their assay in different foods.[19] Both methanol with 0.1% HCl and acetone/water (70/30, v/v) have been used for identification of phenolic compounds in strawberries,[11] and similar extract composition and yield have been obtained, but still more detailed studies have been performed using the acidic methanol solvent because "it is the widely accepted solvent of choice for the extraction of anthocyanins".[14] A more detailed study using solvent mixtures of methanol or acetone and acids has been carried out in order to evaluate the effect of solvents and acids on extraction of anthocyanins from strawberry fruits,[20] and acetone has been proposed as giving efficient, more reproducible extraction, avoiding problems with pectins and allowing sample concentration at much lower temperature.

In summary, the wide variety of polyphenols in plants implies the need for establishing effective methodology for their efficient extraction and characterization. In that direction, the aim of the present study was to examine the efficiency of extraction of polyphenols in solvent mixtures containing methanol or acetone in combination with different acids (formic, acetic, hydrochloric acid) in order to evaluate the effect of the extraction solvent composition on the yield of different classes of polyphenols and to find out the most efficient one. Cultivated strawberries from

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Table 1. Contents (Expressed as mg/kg Fresh Fruit), Retention Times and MS Spectral Data of Anthocyanins, Flavonols and Hydroxycinnamic Acid Derivatives Measured in Strawberry Extracts Obtained with Eight Solvent Mixtures (Mean of Three Replicates ± Standard Deviation)

	compounds	t_R/min	MS (m/z)	ext 1[a]	ext 2	ext 3	ext 4	ext 5	ext 6	ext 7	ext 8
						Anthocyanins					
A1	Cy-3-glucoside	25.85	449 $[M]^+$	23.6 ± 1.0	23.4 ± 1.5	22.5 ± 4.2	22.7 ± 2.1	23.8 ± 0.9	22.2 ± 2.7	22.2 ± 4.1	27.0 ± 0.3
A2	Plg-3-glucoside[b]	27.35	433 $[M]^+$	69.0 ± 7.3 adg	57.1 ± 1.7 cdf	46.9 ± 9.2 bdf	56.6 ± 0.4 abcf	84.6 ± 3.6 g	50.6 ± 8.5b cd	42.7 ± 8.0 bcdf	76.9 ± 4.5 aeg
A3	Plg-3-rutinoside	28.15	579 $[M]^+$	32.0 ± 1.6	33.2 ± 3.9	30.3 ± 7.5	31.6 ± 4.2	31.9 ± 1.5	30.5 ± 3.8	30.3 ± 7.0	32.5 ± 1.3
A4	Plg-3-malonylglucoside	31.21	519 $[M]^+$	25.8 ± 6.0	34.7 ± 8.3	29.6 ± 0.9	30.6 ± 2.3	36.6 ± 2.5	30.3 ± 5.6	29.0 ± 1.2	40.3 ± 1.9[c]
A5	Plg-3-acetylglucoside	33.52	475 $[M]^+$	27.2 ± 0.6	23.0 ± 1.1	22.6 ± 0.4	22.9 ± 0.7	23.5 ± 0.7	22.7 ± 0.2		
total anthocyanins[b]				177.6 ± 1.1 b	171.4 ± 3.0 b	151.9 ± 3.9 b	164.5 ± 1.7 b	200.3 ± 1.5a	156.2 ± 3.5 b	124.3 ± 4.1 c	176.7 ± 1.8 ab
						Flavonols					
F1	Q-3-glucuronide	32.20	477 $[M-H]^-$	19.2 ± 5.9	15.3 ± 0.7	13.8 ± 1.3	14.0 ± 0.0	12.4 ± 0.6	11.9 ± 0.3	12.0 ± 0.1	12.8 ± 0.1
F2	K-3-coumaroylglucoside	35.30	593 $[M-H]^-$	16.0 ± 1.6	14.5 ± 1.7	13.9 ± 1.5	14.4 ± 3.7		13.2 ± 0.1	13.4 ± 1.4	14.0 ± 0.7
F3	K-3-acetylglucoside	36.15	489 $[M-H]^-$	9.2 ± 0.2	12.1 ± 1.4		11.6 ± 0.8	17.1 ± 1.1		11.2 ± 0.2	12.3 ± 0.1
total flavonols[b]				44.3 ± 2.41 a	41.9 ± 1.4 a	27.7 ± 1.0 bc	40.0 ± 1.6 ab	29.5 ± 0.5bc	25.1 ± 0.2 c	36.7 ± 0.6 ab	39.7 ± 0.3 ab
						Hydroxycinnamic Acid Derivatives					
H1	caffeoylhexose	19.26	341 $[M-H]^-$	5.9 ± 0.3	6.6 ± 0.2	6.5 ± 0.1	6.7 ± 0.5	6.8 ± 0.3	6.0 ± 0.0	6.6 ± 0.6	6.4 ± 0.2
H2	*p*-coumaroylhexose	22.41	325 $[M-H]^-$	8.7 ± 0.6	14.1 ± 0.8	13.8 ± 1.1	18.8 ± 6.1	15.5 ± 1.3	6.1 ± 0.7	10.4 ± 0.6	12.2 ± 0.1
H3	*p*-coumaroylhexose	23.67	325 $[M-H]^-$	5.8 ± 0.3	7.1 ± 0.2	7.5 ± 0.2	8.7 ± 0.4	6.8 ± 0.4	9.6 ± 0.1	6.8 ± 0.7	7.4 ± 0.4
H4	ferulic acid hexose derivative	27.03	449 $[M-H]^-$		1.1 ± 0.0	0.3 ± 0.0	3.1 ± 0.0		3.4 ± 0.0	8.3 ± 0.9	
total hydroxycinnamic acid deriv[b]				20.3 ± 0.2 a	28.9 ± 0.3 c	28.2 ± 0.4 c	37.3 ± 2.5 b	29.1 ± 0.6 c	25.1 ± 0.1 c	32.1 ± 0.5 c	25.9 ± 0.2 c

[a] Ext 1: acetone. Ext 2: acetone/acetic acid (99:1, v/v). Ext 3: acetone/formic acid (99:1, v/v). Ext 4: acetone/water/acetic acid (70:29.5:0.5, v/v/v). Ext 5: methanol. Ext 6: methanol/acetic acid (99:1, v/v). Ext 7: methanol/formic acid (95:5, v/v). Ext 8: methanol/water/HCl (80:18:2, v/v/v). Cy: cyanidin. Plg: pelargonidin. Q: quercetin. K: kaempferol. [b] Means ($n = 3$) ± SD in the same row followed by same letters are not significantly different by Newman−Keuls test ($p < 0.05$). [c] Not significantly different at $p < 0.05$, significantly different at $p < 0.01$ (Newman−Keuls test).

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Table 2. Contents (Expressed as mg/kg Fresh Fruits), Retention Times and MS Spectral Data of Flavan-3-ols, Conjugated Forms of Ellagic Acid and Total Phenolic Compounds Measured in Strawberries Extracts Obtained with Eight Solvent Mixtures (Mean of Three Replicates ± Standard Deviation)

	compounds	t_R/min	MS (m/z)	ext 1[a]	ext 2	ext 3	ext 4	ext 5	ext 6	ext 7	ext 8
						Flavan-3-ols					
P1	proanthocyanidin dimer	17.91	577 $[M-H]^-$	23.1 ± 1.3	37.5 ± 5.3	19.0 ± 2.1	27.3 ± 4.6	9.7 ± 0.6	6.3 ± 0.4	3.2 ± 0.0	11.0 ± 1.0
P2	proanthocyanidin tetramer	18.37	1153 $[M-H]^-$	5.0 ± 1.0	36.0 ± 3.2	44.7 ± 7.0	26.1 ± 1.2	3.4 ± 0.1	6.6 ± 0.2	0.9 ± 0.0	4.1 ± 0.9
P3	proanthocyanidin tetramer	18.61	1153 $[M-H]^-$		43.6 ± 2.1	46.4 ± 3.2				5.7 ± 0.4	
P4	proanthocyanidin tetramer[b]	19.65	1137 $[M-H]^-$	2.0 ± 0.4	23.7 ± 2.2	11.7 ± 0.6		1.2 ± 0.2			
P5	proanthocyanidin trimer	20.03	849 $[M-H]^-$	0.7 ± 0.0	15.2 ± 1.3			1.0 ± 0.1	2.8 ± 0.0		
P6	proanthocyanidin dimer	20.20	561 $[M-H]^-$	33.6 ± 2.6	13.7 ± 2.8	9.2 ± 0.6	9.2 ± 0.7	21.9 ± 0.2			
P7	catechin	20.54	289 $[M-H]^-$	81.5 ± 2.4	73.8 ± 8.5	86.0 ± 10.0	37.3 ± 5.4	73.1 ± 0.2	12.8 ± 0.7	12.3 ± 0.4	40.1 ± 3.8
P8	proanthocyanidin dimer	21.39	577 $[M-H]^-$	2.5 ± 0.5	36.8 ± 3.5	45.2 ± 12.2	53.3 ± 5.0	3.7 ± 0.3			14.2 ± 1.3
P9	proanthocyanidin trimer	21.76	865 $[M-H]^-$	3.1 ± 0.7	41.7 ± 2.5		33.7 ± 5.5	4.2 ± 0.4			
P10	proanthocyanidin trimer	24.21	849 $[M-H]^-$		14.8 ± 0.1						14.9 ± 3.6
P11	proanthocyanidin trimer	25.62	849 $[M-H]^-$		30.3 ± 3.8						
total flavan-3-ols				151.4 ± 0.2	367.1 ± 2.7	262.1 ± 4.7	187.0 ± 3.0	118.1 ± 0.2	28.5 ± 0.3	22.2 ± 0.2	84.3 ± 1.9
						Conjugated Forms of Ellagic Acid					
E1	*bis*-HHDP-glucoside	15.84	783 $[M-H]^-$	7.8 ± 0.2	7.6 ± 0.4			4.2 ± 0.4			
E2	galloyl-*bis*-HHDP-glucoside	22.62	935 $[M-H]^-$	5.2 ± 0.1	14.9 ± 2.3	42.1 ± 9.3	21.0 ± 1.3	5.4 ± 0.4			
E3	galloyl-*bis*-HHDP-glucoside	23.02	935 $[M-H]^-$	18.8 ± 1.0	21.6 ± 1.6			14.9 ± 1.5			
E4	dimer of galloyl-*bis*-HHDP -glucoside (sanguiin H-6)	24.49	934 $[M-H]^{2-}$	96.6 ± 2.1	185.3 ± 14.4	161.2 ± 10.0	53.8 ± 2.8	137.9 ± 5.2			
E5	ellagic acid deoxyhexoside	32.91	447 $[M-H]^-$	5.9 ± 0.1	5.3 ± 0.1	5.9 ± 0.1	3.4 ± 0.8	5.6 ± 0.2	1.1 ± 0.0	1.2 ± 0.0	3.5 ± 0.3
total conjugated forms of ellagic acid				134.4 ± 0.9	234.7 ± 4.6	209.2 ± 5.6	78.2 ± 1.3	168.0 ± 1.2	1.1 ± 0.0	1.2 ± 0.0	3.5 ± 0.3
total phenolic compounds				528.0 ± 1.5	844.0 ± 1.6	678.9 ± 1.8	506.9 ± 1.4	573.1 ± 1.0	236.0 ± 0.9	216.4 ± 1.3	329.4 ± 0.9

[a] Ext 1: acetone. Ext 2: acetone/acetic acid (99:1, v/v). Ext 3: acetone/formic acid (99:1, v/v). Ext 4: acetone/water/acetic acid (70:29.5:0.5, v/v/v). Ext 5: methanol. Ext 6: methanol/acetic acid (99:1, v/v). Ext 7: methanol/formic acid (95:5, v/v). Ext 8: methanol/water/HCl (80:18:2, v/v/v). HHDP: hexahydroxydiphenoyl. [b] One (epi)afzelechin unit is present in the proanthocyanidin oligomer (only (epi)catechin units in the others).

the *Maya* variety (*Fragaria ananassa* species) were used as a model sample since their phenolic profile has been previously investigated in our laboratory and found to be rich in polyphenols from the five classes: anthocyanins, flavonols, flavan-3-ols, hydroxycinnamic acid derivatives and ellagic acid derivatives.[13] The particular interest was to test various combinations of methanol or acetone and acids (formic, acetic, hydrochloric), some already used by other researchers and some tested in our laboratory, in order to establish the most efficient solvent mixture for extraction of all classes of polyphenols with regard to both the number of compounds and their quantity.

MATERIALS AND METHODS

Plant Material. The variety *Maya* from *Fragaria ananassa* species has been introduced as a promising cultivar, and a demonstrative orchard was established in September 2006, in the region of Kočani (Teranci), in east Macedonia, with imported green seedlings rooted in pots. The orchard has been established in a system of two-row beds with a width of 80 cm, mulched with black foil. The distance between seedlings in the beds was 40 × 30 cm, with an empty space of 60 cm between the beds. In order to enforce the production of fruits, in spring, the orchard was covered with high polyethylene tunnels. The strawberries were harvested at commercial ripeness, specifically when 80% of the surface was red, which corresponds to stage 5 in terms of commercial criterion. The strawberries were harvested on May 08 2009, 500 g was randomly sampled and samples were stored at −80 °C until analysis.

Sample Preparation. Phenolic compounds were extracted from 5 g of frozen strawberry samples (three replicates from each sample) in 10 mL of extraction solvent mixture. Eight different solvent mixtures containing methanol or acetone in combination with different acids (formic, acetic, hydrochloric acid) were tested: acetone (extraction 1); acetone/acetic acid (99:1, v/v), (extraction 2); acetone/formic acid (99:1, v/v) (extraction 3); acetone/water/acetic acid (70:29.5:0.5, v/v/v) (extraction 4);[12,19,20] methanol (extraction 5); methanol/acetic acid (99:1, v/v) (extraction 6); methanol/formic acid (95:5, v/v) (extraction 7);[21] and methanol/water/HCl (80:18:2, v/v/v) (extraction 8).[16]

All extracts were sonicated for 15 min then centrifuged for 15 min at 3000 rpm, and the supernatants were concentrated in rotary-evaporator at low temperature (37 °C) to yield aqueous residue. pH values of the solvent extraction mixture, fresh extract and the aqueous residue after evaporation were measured. The sample was then diluted to 10 mL with 20% methanol, and it was filtered through 0.45 μm pore-size polyethersulfone filter (Econofilter, 25/0.45 μm NL, Agilent Technologies, Germany) before analysis. All extracts were analyzed by HPLC−DAD−ESI-MS. Extractions and analyses were made in triplicate.

Reagents and Standards. Water, hydrochloric acid, formic acid and methanol all of analytical grade were purchased from Merck KGaA (Darmstadt, Germany). Acetone and acetic acid were purchased from Alkaloid (Skopje, Macedonia). Pelargonidin-3-glucoside and proanthocyanidin dimer B2 were from Phytolab (Vestenbergsgreuth, Germany), catechin and quercetin were from Sigma (Germany), and *p*-coumaric, ferulic and ellagic acid were from Extrasynthese, (Genay, France).

Standard solutions in the concentration ranges of 100−500 μM for pelargonidin-3-glucoside and *p*-coumaric acid, and from 10 to 50 μM for catechin, proanthocyanidin dimer B2, quercetin, *p*-coumaric acid, ferulic

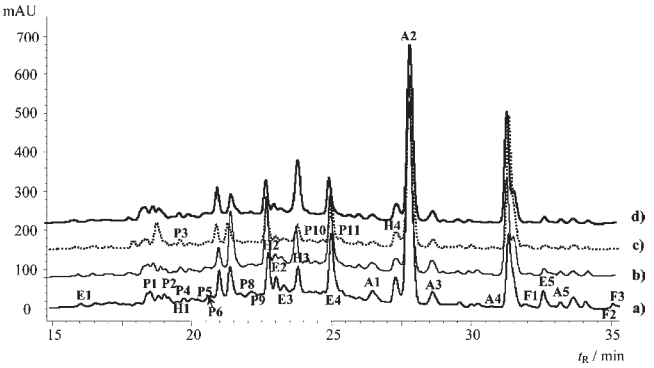

Figure 1. HPLC−DAD chromatograms recorded at 280 nm corresponding to extracts obtained with (a) acetone (extraction 1); (b) acetone/acetic acid, 99:1, v/v (extraction 2); (c) methanol (extraction 5); (d) methanol/acetic acid, 99:1, v/v (extraction 6). Peaks' annotations correspond to ones in the first column in Tables 1 and 2.

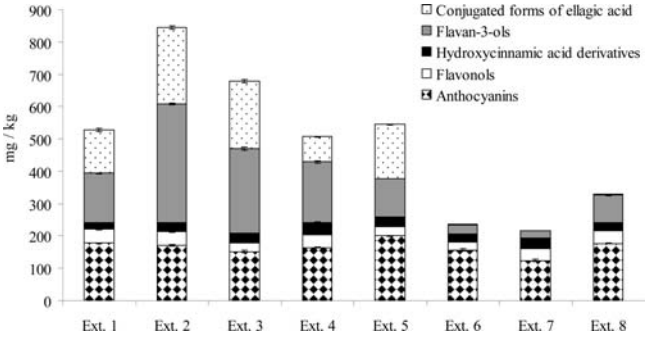

Figure 2. Total content of phenolic compounds with contributions from each group (expressed as mg/kg fresh fruit) in the extracts: Ext 1: acetone. Ext 2: acetone/acetic acid (99:1, v/v). Ext 3: acetone/formic acid (99:1, v/v). Ext 4: acetone/water/acetic acid (70:29.5:0.5, v/v/v). Ext 5: methanol. Ext 6: methanol/acetic acid (99:1, v/v). Ext 7: methanol/formic acid (95:5, v/v). Ext 8: methanol/water/HCl (80:18:2, v/v/v).

acid, and ellagic acid were used for calibration. Peak areas were used for quantitation at wavelengths where each group of phenolic compounds exhibits an absorption maximum.

LC−DAD−ESI-MS^n Analysis. Chromatographic separations were carried out on 150 mm × 4.6 mm, 5 μm Eclipse XDB-C18 column (Agilent, Germany). The mobile phase consisted of two solvents: water−formic acid (99/1, v/v) (A) and methanol (B). A linear gradient starting with 5% B (0−5 min) was set to reach 80% B at 45 min, 100% B at 50 and hold for 10 min. The flow rate was 0.4 mL min^{-1} and the injection volume 20 μL.

The HPLC system was from Agilent 1100 series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser, G1315B photodiode array detector and G2445A ion-trap mass spectrometer, controlled by ChemStation (Agilent, v.01.03) and LCMSD software (Agilent, v.6.2). Spectral data from all peaks were accumulated in the range 190−600 nm, and chromatograms were recorded at 260 nm for ellagic acid and its conjugated forms, 280 nm for flavan-3-ols and their dimers, trimers and tetramers, 320 nm for conjugated forms of hydroxycinnamic acids, 360 nm for flavonol glycosides and 520 nm for anthocyanins.

The ion-trap mass spectrometric detector (Agilent G2445A) was equipped with an electrospray ionization (ESI) system and controlled by LCMSD software. Nitrogen was used as nebulizing gas at pressure of 50 psi, and the flow was adjusted to 12 L min^{-1}. The heated capillary and the voltage were maintained at 325 °C and 3.5 kV, respectively. MS data were acquired in positive and negative ionization mode. The full scan covered the mass range from m/z 100−2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of the ion trap and the number of MS repetitions to obtain the MS average spectra was set at 200 ms and 5, respectively.

Table 3. pH Values of (a) Solvent Mixtures, (b) Fresh Extracts, and (c) Aqueous Residues (after Evaporation)

		pH		
	solvent	a	b	c
1	acetone	6.3	4.8	3.7
2	acetone/acetic acid (99:1)[a]	5.4	4.7	3.8
3	acetone/formic acid (99:1)[a]	4.5	4.0	3.5
4	acetone/water/acetic acid (70:29.5:0.5)[a]	4.0	3.7	3.4
5	methanol	5.5	5.0	4.0
6	methanol/acetic acid (99:1)[a]	4.7	4.2	3.7
7	methanol/formic acid (95:5)[a]	4.0	3.6	3.0
8	methanol/water/HCl (80:18:2)[a]	3.5	3.3	2.7

[a] Volume fractions.

Statistical Analysis. Statistical treatment including calculations of means and standard deviations were performed applying Excel (Microsoft Office, 2003). Samples were analyzed in triplicate, and one-way analysis of variance (ANOVA) was performed using STATISTICA, version 7. The Newman−Keuls post hoc test (at $p < 0.05$ and 0.01) was used to determine the significant differences between different extractions.

RESULTS AND DISCUSSION

Five groups of phenolic compounds, namely, anthocyanins, flavonols, hydroxycinnamic acid derivatives, flavan-3-ols and conjugated forms of ellagic acid, were analyzed in the prepared 8 types of extracts. Identification of phenolic compounds was done by retention times of peaks in chromatograms and with their corresponding UV−vis absorption and mass spectral data. A detailed characterization of the mass spectra of all detected phenolic compounds is given and available elsewhere.[13] Since the main goal of this study was to test efficiency of different solvents on extraction of total phenolic compounds, eight different extraction mixtures were established (given in Materials and Methods, Sample Preparation), three of which (extraction 2, 3 and 6) were the ones used in our lab,[13,22] and the other three have been used by other authors for studies of phenolic compounds: extraction 4,[12,19,20] extraction 7,[21] and extraction 8.[16] Extractions in pure acetone and methanol (extraction 1 and extraction 5, respectively) have been performed in order to evaluate the effect of the acids on the extraction efficiency of various classes of polyphenols.

The data corresponding to the amounts of 28 phenolic compounds determined by HPLC−DAD−ESI-MS in the extracts obtained using these eight different solvent extraction mixtures are summarized in Table 1 for anthocyanins, flavonols and hydroxycinnamic acid derivatives and in Table 2 for flavan-3-ols and conjugated forms of ellagic acid. The chromatograms in Figure 1 illustrate the differences between the corresponding extracts obtained in pure acetone and methanol and in mixtures of these solvents with acetic acid (99:1, v/v). For a more clear view of the obtained yield of phenolic compounds, the total

content of phenolic compounds and the contribution of each group (summed individual contents determined by HPLC−DAD) in each extract are presented in Figure 2. The graphical presentation clearly indicates the differences between the various solvents used, which is also supported by a statistical analysis (Newman−Keuls test at $p < 0.05$ and also $p < 0.01$).

Acetone containing mixtures (extraction 1−4) gave much higher yields of total phenolic compounds than methanol containing ones (extraction 5−8), which can mainly be attributed to the higher quantities of flavan-3-ols (proanthocyanidins) and conjugated forms of ellagic acid, whereas comparable yields have been obtained for anthocyanins, flavonols and hydroxycinnamic acid derivatives.

The results for total anthocyanins were comparable to those for all 8 studied extraction solvents, but extraction 5 (methanol) produced significantly higher yield and extraction 7 (methanol/formic acid, 95:5, v/v) significantly lower yield (at $p < 0.05$). As can be seen from the results, the highest variations were found in the methanol containing solvent mixtures and acidic methanol as a solvent was found as not very suitable for extraction of anthocyanins. Moreover, in the extracts obtained with solvent mixtures of methanol with 5% formic acid (extraction 7) and methanol/water/HCl 80:18:2, v/v/v (extraction 8), the acetylglucoside of pelargonidin was not detected, implying its hydrolysis. The pH values were measured for all solvent extraction mixtures, freshly prepared extracts and the aqueous residues after evaporation (Table 3). The values obtained for extracts 7 and 8 after evaporation are the lowest (pH ≤ 3), implying the hydrolysis of the acetyl moiety, which is evident for extract 8 with significantly higher content of pelargonidin-3-*O*-glucoside compared to the other extracts (Table 1). Acetylation and formylation of anthocyanin sugars when using organic acids in the extraction solvent have been demonstrated,[23] and this possibility together with the problems associated with the handling of the extract (filtration through 0.45 μm) could be avoided by using acetone as an extraction solvent as suggested earlier.[20] Moreover, in this work, acetone and its mixtures with acids were found to be not statistically different in the extraction efficiency of anthocyanins, implying that the presence and nature of the acid do not significantly affect the extraction of anthocyanins in acetone.

Acetone has been shown as a better extraction solvent for flavonols, as well, since significant differences were found for the yield of total flavonols in acetone compared to methanol ($p < 0.05$). It is interesting that very close contents of flavonols, and statistically not different amounts of the specific flavonols, were measured in aqueous acidic acetone (extraction 4) and methanol (extraction 8) implying that acidified water in acetone and methanol perform as equivalent solvent mixtures for extraction of flavonols.

As for hydroxycinnamic acid derivatives, the acetone/water/acetic acid mixture (extraction 4) was found as the most efficient and pure acetone as the least efficient (at $p < 0.05$), whereas the extraction mixtures containing methanol were comparable between themselves and the acidic nonaqueous acetone containing mixtures. In spite of the highest efficiency, nonaqueous solvent mixtures can be recommended because of obtaining more clear extracts after centrifugation and more convenient procedure for vacuum rotary evaporation (37 °C). Also, weak organic acids instead of hydrochloric acid should be used because of the possibility of hydrolysis and decomposition and also the formation of insoluble colored decomposition products as previously suggested.[20,23]

The most significant differences, as shown in Figure 2, were found in the extraction efficiency of flavan-3-ols and conjugated forms of ellagic acid. Acetone was proved as superior solvent for extraction of flavan-3-ols (Table 2 and Figure 2) since lower contents of catechin and especially proanthocyanidin oligomers (dimers, trimers and tetramers of (epi)catechin and (epi)afzelechin) were detected and measured in methanol based extraction solvents, especially in the acidic nonaqueous ones (extraction 6 and 7). On the other hand, acetone−acid mixtures were more efficient than pure acetone. These results suggest acetone and acetic acid, 99:1 v/v (extraction 2), as a superior extraction solvent for catechin and proanthocyanidin oligomers since it extracts the highest quantity, but also quality i.e. number of proanthocyanidin oligomers in the extracts and would be most appropriate for studies of the nature and content of these compounds.

Analogous results have been obtained for the conjugated forms of ellagic acid (*bis*-HHDP-glucose, two isomers of galloyl-*bis*-HHDP-glucose, sanguiin H-6, and ellagic acid deoxyhexoside). All five compounds were detected in pure methanol (extraction 5), pure acetone (extraction 1) and acetone−acetic acid mixture (extraction 2) and only the last compound, deoxyhexoside of ellagic acid, was detected in all extracts. Acidified methanol was shown as a very pure extraction solvent for this group of compounds, pure methanol significantly more efficient, but acetone−acid mixtures (nonaqueous) were superior to the others, especially the acetone−acetic acid, 99:1 v/v (extraction 2), followed by acetone-formic acid, 99:1 v/v (extraction 3). 70% acetone in water and 80% methanol in water have previously been compared for extraction of phenolics from strawberries, and it has been concluded that acetone extracts contain more polyphenols especially ellagic acid derivatives.[10] The results obtained in this survey imply nonaqueous acetone containing acetic acid (similarly with formic acid) as the most effective extraction solvent for this group of phenolic compounds, but analogous methanol containing solvent mixtures as totally inefficient.

Studying the graph in Figure 2, the following conclusions can be made:

1. Pure acetone is an efficient extraction solvent for anthocyanins and flavonols, with intermediate efficiency for flavan-3-ols, procyanidins and ellagic acid derivatives and a poor extraction solvent for hydroxycinnamic acid derivatives. The comparison of pure acetone and pure methanol reveals a significant difference between them for the extraction efficiency of all studied groups at $p < 0.05$, methanol being a better solvent for anthocyanins and hydroxycinnamic and ellagic acid derivatives, but acetone for flavonols and flavan-3-ols. However, with a more rigorous statistical treatment ($p < 0.01$) the difference is significant only for extraction of the derivatives of ellagic acid.
2. Addition of acetic or formic acid to acetone gives similar efficiency for extraction of anthocyanins and flavonols (significantly lower for acetone−formic acid mixture), slightly better efficiency for hydroxycinnamic acid derivatives (significant at $p < 0.05$) and much better efficiency for flavan-3-ols and ellagic acid derivatives ($p < 0.01$). The extraction mixture 4 containing also water (acetone:water:acetic acid, 70:29.5:0.5, v/v/v), which has been used for proanthocyanidin oligomers and other phenolics in strawberries,[10] and ellagitannins in blackberries,[24] gave significantly lower yields of flavan-3-ols and conjugated

forms of ellagic acid compared to nonaqueous acidified acetone (extraction 2 and 3), implying the negative effect of water in the extraction solvent on these compounds, whereas similar content was measured for anthocyanins and flavonols and slightly higher of hydroxycinnamic acid derivatives.

3. Among methanol containing extraction solvents, pure methanol gave the highest yield of total phenolics. It was most efficient for extraction of anthocyanins of all eight tested solvents and comparable to acetone for ellagic acid derivatives. Among the methanol containing extraction solvents, pure methanol gave highest content of total phenolic compounds and significantly higher yield of flavan-3-ols and especially ellagic acid derivatives compared to acidic methanol solvents. The extraction efficiencies for anthocyanins, flavonols and hydroxycinnamic acid derivatives were comparable to those obtained with acetone containing extraction mixtures.

The results from this study suggest acetone−acetic acid mixture (99:1, v/v) as the most effective solvent for extraction of the various classes of polyphenols in strawberries. The highest yield and also variety of the different polyphenols obtained in a single extraction step together with the already established convenience[20] for the further HPLC analysis of nonaqueous acetone extracts reveal this procedure as most suitable for qualitative and quantitative assays of polyphenols in strawberries that can also be used for studies of polyphenols in other plant samples, especially in berry fruits as already applied in our laboratory for studies of the polyphenol profiles of blueberries and red and black currants.[22] The significantly better extraction efficiency of this solvent for flavan-3-ols and proanthocyanidins is in favor of improving the ratio of extractable to nonextractable polyphenols since it has been shown that usually the polyphenol contents in plant foods are underestimated due to the significant amounts of polyphenols that remain in the residue from extraction mainly due to the nonextractable procyanidins and hydrolyzable polyphenols.[25] Our results can contribute to the selection of the most efficient extraction solvents to be used when analyses of total extractable polyphenols or specific groups of polyphenols are to be made.

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