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CHARACTERIZATION AND QUANTIFICATION OF FLAVONOID AGLYCONES AND PHENOLIC ACIDS IN THE HYDROLYZED METHANOLIC EXTRACT OF *Caucalis platycarpos* USING HPLC-DAD-MS/MS

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*The water extract of burr parsley (*Caucalis platycarpos *L.) showed remarkable antitumor activity in rats and mice. Phenolic compounds, including phenolic acids and flavonoids, are considered to be the major bioactive compounds. The aim of this work was to develop a reverse phase HPLC-DAD method for the simultaneous quantification of flavonoid aglycones and phenolic acids, and an HPLC-DAD-MS/MS method for structural characterization of phenolic compounds, obtained after hydrolysis of* C. platycarpos *methanolic extract. Caffeic acid was the predominant phenolic acid, and luteolin was the predominant flavonoid aglycone. The optimized and validated method for the determination of the five phenolic acids and four flavonoid aglycones ensured reliable results and could be used for the quality control of raw plant material.*

Keywords: *Caucalis platycarpos* L., flavonoid aglycones, phenolic acids, HPLC-DAD-MS.

Naturally occurring immunological response modifiers in combination with cytostatics are widely used in cancer treatment [1, 2]. Burr parsley (*Caucalis platycarpos* L., *Caucalis daucoides* L., Apiaceae), grows in Mediterranean and Central Europe. It has been shown that the water decoction of *C. platycarpos* after intraperitoneal injection had antitumor activity on the rat model of liver metastasis of colorectal cancer. The antitumor effect results from stimulation of the immunological system of the host, by involving the spleen as a lymphatic organ to produce antitumor factors [3]. Flavonoids and phenolic acids as ubiquitous secondary plant metabolites are distributed in nature in their free and bound forms [4]. Luteolin-7-*O*-glucoside was found to be the predominant flavonoid and 3-*O*-caffeoylquinic acid was the predominant phenolic acid in *C. platycarpos* methanolic extract, where 31 compounds were identified [5]. The goal of this work was to develop an HPLC-DAD-MS/MS method for structural characterization and identification of phenolic compounds obtained after hydrolysis of methanolic extract and to develop and validate the HPLC-DAD method for the determination of aglycones and phenolic acids.

The hydrolysis conditions of phenolic acid esters and flavonoid glycosides were optimized in terms of duration of hydrolysis and temperature and concentration of hydrochloric acid [6]. Different conditions were examined by recording the total ion chromatogram and extracted ion chromatogram at *m/z* 447, which is the $[M - H]^-$ for the main flavonoid glycoside present in *C. platycarpos*, namely luteolin-7-*O*-glucoside. Hydrolysis was considered to be completed when there was no peak assigned with *m/z* 447 in the hydrolyzed methanolic extract. BHT at concentration 1.5 g/L was added as antioxidant. It was found that optimum hydrolysis conditions were in 1.5 M HCl for 3 h on boiling water bath, protected from light. Under these conditions, small amounts of 3-*O*-caffeoylquinic acid and 5-*O*-feruloylquinic acid are not completely hydrolyzed. By increasing the concentration of hydrochloric acid and duration of hydrolysis, degradation of phenolic components of interest and formation of quinones occur.

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TABLE 1. Retention Time (T_R), Linear Regression, LOQ, LOD, and Precision Data

Compound	T_R, min	Regression equation[a]	Linear range, μg/mL	LOD, μg/mL	LOQ, μg/mL	Precision (RSD %)				
						conc., μg/mL	intraday		interday	
							T_R	AUC	T_R	AUC
1	8.468 ± 0.029	y = 0.015x–0.048	5–125	2.03	4.13	5.0	0.11	1.83	0.40	2.01
						20.0	0.53	0.95	0.56	1.01
						80.0	0.58	0.54	0.66	1.03
2	12.465 ± 0.037	y = 0.029x–0.069	5–125	2.02	4.11	5.0	0.32	0.99	0.40	1.14
						20.0	0.39	0.82	0.41	1.21
						80.0	0.29	0.80	0.50	1.30
3	18.175 ± 0.054	y = 0.025x–0.029	5–100	1.36	4.14	5.0	0.13	1.02	0.29	1.91
						20.0	0.18	1.18	0.24	1.36
						80.0	0.25	0.41	0.28	1.31
4	19.585 ± 0.054	y = 0.028 x–0.068	5–130	1.17	3.54	5.0	0.15	1.06	0.24	1.33
						20.0	0.30	1.10	0.39	1.27
						80.0	0.31	0.45	0.33	0.65
5	21.542 ± 0.036	y = 0.028x–0.079	5–160	1.23	4.96	5.0	0.19	1.27	0.27	1.39
						20.0	0.53	0.84	0.54	0.77
						80.0	0.50	0.43	0.56	0.48
6	34.903 ± 0.107	y = 0.022 x–0.052	5–130	1.25	3.80	5.0	0.14	1.58	0.24	1.80
						20.0	0.37	1.04	0.45	1.25
						80.0	0.22	0.74	0.30	1.39
7	37.620 ± 0.071	y = 0.022 x–0.042	5–125	0.98	2.97	5.0	0.11	1.08	0.26	1.13
						20.0	0.18	0.96	0.21	1.09
						80.0	0.26	0.58	0.38	0.64
8	42.281 ± 0.119	y = 0.021 x–0.009	5–125	1.50	4.55	5.0	0.14	1.04	0.25	0.98
						20.0	0.10	1.09	0.24	1.34
						80.0	0.11	1.18	0.35	1.19
9	42.784 ± 0.121	y = 0.026 x–0.009	3–80	0.94	2.85	3.0	0.14	1.38	0.23	1.56
						10.0	0.18	0.97	0.24	1.01
						40.0	0.26	0.65	0.33	1.57

[a]Linear regression equation y = ax + b, in which x is the concentration in μg/mL and y is the peak area at the selected wavelength.

Correlation coefficient for **1**, **2**, **7**, **9** – $R^2 = 0.9998$, for **3**, **4**, **5** – $R^2 = 0.9996$, and for **6**, **8** – $R^2 = 0.9999$.

AUC, area under curve.

A method coupling high-performance liquid chromatography (HPLC) with diode-array detector (DAD) and electrospray ionization mass spectrometry with an ion trap analyzer was optimized for the separation and identification of flavonoid aglycones and phenolic acids in the hydrolyzed methanolic extract of *C. platycarpos*. To obtain chromatograms with good resolution within an acceptable time of analysis and not to interfere with ionization of molecules of interest, different mobile phases were tested. 10 mM ammonium formate in water–methanol 85:15, v/v, pH 4.0 adjusted with formic acid, as solvent A and 10 mM ammonium formate in water–methanol 15:85, v/v, pH 4.0 adjusted with formic acid, as solvent B were chosen as suitable for the gradient elution. The presence of acid in the mobile phase provided better separation and retention of phenolic compounds on reverse phase packing and prevented peak tailing of these compounds. Acetic acid had a weak ion-pairing capacity, so the 10 mM ammonium formate was used to buffer the mobile phase at pH 4.0.

The UV absorption maxima of most flavonoids and phenolic acids (310, 325, 350 and 370 nm) were chosen as monitoring wavelengths. Apigenin and chrysoeriol were found to be eluted closely, but the differences in their absorption spectra were utilized for their quantification by comparing their chromatograms recorded at 325 nm, for apigenin, and 350 nm, for chrysoeriol.

MS analysis in the negative ion mode of ESI provided extensive structure information for most flavonoids and phenolic acids present in *C. platycarpos*. In addition to detection of the deprotonated molecular ions, collision induced dissociation (CID) was performed in the MS^2, and the resulting product ions were used as fingerprints of each component.

TABLE 2. Recovery and Accuracy Data, %

Amount added, μg/mL	Recovery	RSD	Recovery	RSD	Recovery	RSD
	3-*O*-Caffeoylquinic acid (**1**)		Caffeic acid (**2**)		*p*-Coumaric acid (**3**)	
6.7	98.53	0.90	98.68	1.70	101.60	1.83
26.7	100.33	0.44	99.74	0.43	100.53	0.46
41.7	99.41	0.90	99.29	0.26	100.42	1.15
	Ferulic acid (**4**)		Isoferulic acid (**5**)		Quercetin (**6**)	
6.7	99.31	0.62	99.47	0.55	98.80	1.17
26.7	101.15	0.38	100.90	0.60	99.44	1.02
41.7	98.87	1.11	100.72	0.62	100.14	0.45
	Luteolin (**7**)		Apigenin (**8**)		Chrysoeriol (**9**)	
6.7	98.57	1.52	101.22	1.08	98.26	1.92
26.7	101.01	1.16	101.18	0.90	100.65	1.28
41.7	99.43	0.31	100.99	0.17	99.56	0.84

TABLE 3. Phenolic Compounds in the Hydrolyzed Methanolic Extract of *C. platycarpos*

Compound	Average amount, (mg/kg dry matter) ± SD	Compound	Average amount, (mg/kg dry matter) ± SD
3-*O*-Caffeoylquinic acid	52.22 ± 0.24	Quercetin	372.16 ± 2.17
Caffeic acid	297.70 ± 2.69	Luteolin	912.64 ± 7.54
p-Coumaric acid	68.03 ± 1.24	Apigenin	70.37 ± 0.94
Ferulic acid	66.15 ± 0.80	Chrysoeriol	68.36 ± 1.27
Isoferulic acid	23.91 ± 0.18		

Values are means ± SD, n = 6.

The optimized RP-HPLC-DAD method for the simultaneous analysis of four flavonoid aglycones [quercetin (**6**), luteolin (**7**), apigenin (**8**), and chrysoeriol (**9**)] and five phenolic acids [3-*O*-caffeoylquinic acid (**1**), caffeic acid (**2**), *p*-coumaric acid (**3**), ferulic acid (**4**), isoferulic acid (**5**)] was validated in terms of linearity, limit of detection, limit of quantification, precision, and accuracy.

Chromatogram peak areas at 310 nm for *p*-coumaric acid, 325 nm for **1**, **2**, **4**, **5**, and **8**, 350 nm for luteolin (**7**) and chrysoeriol (**9**), and 370 nm for quercetin (**6**) were plotted against the known concentrations of the standards to establish calibration equations. The calibration curve was obtained with six levels of concentration of standard mixtures. The linear regression equation was calculated by the least squares method. All the components showed good linearity in a relatively wide range. The detection limit (LOD) and limit of quantification (LOQ) were calculated from the residual standard deviation of the regression line (σ) and the slope (S) as follows: LOD = 3.3 σ/S; LOQ = 10 σ/S.

Three different concentrations covering the linear range were used for intra- and interday precision testing. Data for intraday precision of peak areas and retention times were calculated from six non-consecutive injections performed on three different concentrations on the same day. % RSD (relative standard deviation) for interday precision of peak areas and retention times were obtained from three consecutive injections performed at each concentration on 3 different days. The linear range, regression equation, and correlation coefficient of each analytes, LOD and LOQ values, and interday and intraday precision are summarized in Table 1. The sample solution stored at 4°C was found to be stable for 48 h.

HPLC accuracy was determined by analyzing the recovery of sample extracts spiked with three different standard mixture concentrations in the linear range. Analyte recovery was expressed as the percent mean ratio of the measured added concentration to nominal value. Recovery data showing good accuracy are presented in Table 2.

TABLE 4. Peak Identification with Retention Times (R_T), Molecular Weight (MW), Parent Ion ($[M - H]^-$), MS^2 Fragment Ions, and UV Maximum Band

Compound	R_T, min	MW	$[M - H]^-$ *m/z*	MS^2 fragment ions (fragmentation pattern, I_{rel},%)	λ, nm
Gallic acid	6.575	170.12	168.8	124.8 (M – H – CO_2, b.p.)	273
3-*O*-Caffeoylquinic acid (**1**)	8.501	354.31	353.1	190.9 (b.p.), 178.8 (20)	326
Vanillic acid	10.981	168.15	166.8	122.9 (M – H – CO_2, b.p.)	260
Caffeic acid quinone	12.054	178.16	176.8	146.8 (M – H – H_2CO, b.p.)	350
				118.9 (M – $H_2C_2O_2$, 10)	
Caffeic acid (**2**)	12.463	180.16	178.8	134.9 (M – H – CO_2, b.p.)	324
5-FQA (5-*O*-feruloylquinic acid)	17.901	368.35	367.0	190.8 (b.p.), 172.8 (29), 160.8 (3), 136.8 (10)	325
p-Coumaric acid (**3**)	18.384	164.16	162.7	118.9 (M – H – CO_2, b.p.)	310
Ferulic acid (**4**)	19.641	194.18	192.9	148.5 (M – H – CO_2, b.p.)	325
				133.8 (M – H – CO_2 – CH_3, 64)	
Isoferulic acid (**5**)	21.601	194.18	192.9	177.8 (M – H – CH_3, 30)	325
				160.8 (M – H – CH_3 – H_2O, b.p.)	
				133.8 (M – H – CO_2 – CH_3, 47)	
Unknown	25.702	–	386.9	192.9 (b.p.)	325
Quercetin (**6**)	35.321	302.24	300.9	272.9 (M – H – CO, 7)	256
				256.8 (M – H – CO_2, 8)	371
				228.8 (M – H – CO_2 – CO, 6)	
				178.8 ($^{1,2}A^-$, b.p.)	
				150.8 ($^{1,3}A^-$, 98)	
				120.8 ($^{1,2}B^-$, 3)	
				106.9 ($^{0,4}A^-$, 9)	
Luteolin (**7**)	38.054	286.24	284.9	240.8 (M – H – CO_2, 85)	350
				212.8 (M – H – CO_2 – CO, 56)	
				198.8 (M – H – CO_2 – CO – CH_3, 92)	
				174.8 ($^{0,4}B^-$, b.p.)	
				150.8 ($^{1,3}A^-$, 51)	
				132.8 ($^{1,3}B^-$, 31)	
				106.8 ($^{0,4}A^-$, 8)	
Apigenin (**8**)	42.300	270.23	268.9	251.0 (M – H – H_2O, b.p.)	337
				240.7 (M – H – CO, 84)	
				224.9 (M – H – CO_2, 45)	
				150.8 ($^{1,3}A^-$, 94)	
				124.8 ($^{1,4}A^-$, 25)	
				116.8 ($^{1,3}B^-$, 3)	
Chrysoeriol (**9**)	43.144	300.27	298.9	283.9 (M – H – CH_3, b.p.)	349

b.p.: base peak; I_{rel}, %, relative intensities of peaks for ions in percent of the base peak.

Peak identities were assigned based on retention times, UV spectra of the standard compounds using HPLC-DAD, and by comparing fragment ions obtained by HPLC-ESI-MS/MS with those of the standards and with the literature data. The nine phenolic components were simultaneously determined by means of the external standard method. In some cases, for example chlorogenic acid and isoferulic acid, where the compounds were present in very low concentrations, the sample was concentrated to proper volume in order to match the linear range. Caffeic acid was the predominant phenolic acid, and luteolin was the predominant flavonoid aglycone in *C. platycarpos* hydrolyzed methanolic extract. Data of quantitative analyses are listed in Table 3. As the nonhydrolyzed extract contains several isomers of dicaffeoyquinic acid, it is supposed that some of these quinic acid esters remain partly unhydrolyzed, losing one caffeic acid residue. That is probably the reason why the hydrolyzed extract has a minor content of **1**.

ESI mass spectra were recorded in the negative ion mode. The parent ions $[M - H]^-$ were recognized and fragmented using tandem MS, in order to obtain characteristic ions for peak identification. Peak identities with retention time, fragmentation data, and UV band maximum data are listed in Table 4.

The main groups of chlorogenic acids (CGA) found in the unhydrolyzed methanolic extract of *C. platycarpos* include caffeoylquinic acids, feruloylquinic acids, *p*-coumaroylquinic acids, and mixed diesters of caffeic and ferulic acids with quinic acid. Each of these groups contains several isomers [5]. During hydrolysis, CGA are hydrolyzed, isomerized, and degraded into low molecular weight compounds. Elevated temperature results in transformation of the main part of CGA into low molecular phenolic acids and formation of quinone derivatives such as caffeic acid quinone. In unhydrolyzed methanolic extract, ferulic acid is present in a number of esters. In hydrolyzed extract, isoferulic acid is present in small amounts. This may be accounted for isomerization during hydrolysis of esters. In tandem MS hydroxybenzoic acid and hydroxycinnamic acids showed a loss of CO_2 and production of an ion at $m/z \sim [M - H - 44]^-$. Ferulic acid showed the loss of the CO_2 group, providing an ion at $m/z \sim 148.5$, and successive loss of the methyl radical, giving an ion at $m/z \sim 133.8$. Isoferulic acid also loses CO_2 and methyl radical, giving the fragment ion at $m/z \sim 133.8$, but can be distinguished from isoferulic acid by the quinone anion at $m/z \sim 160.8$, resulting from successive loss of the methyl radical and H_2O. 3-*O*-Caffeoylquinic acid (**1**) and 5-*O*-feruloylquinic acid (5-FQA) remain partly unhydrolyzed. The parent ion identifies the CGA subclass, and MS^2 ensures unequivocal identification. Compound **1** showed the $[M - H]^-$ deprotonated molecule at m/z 353.1 and MS^2 ions corresponding to deprotonated quinic acid at $m/z \sim 190.9$, and **2**, at $m/z \sim 178.8$. 5-FQA gives the parent ion at $m/z \sim 367.0$ with the main MS^2 fragment ion at $m/z \sim 190.8$ and secondary peak ions at $m/z \sim 172.8$, 160.8, and 134.8 [4, 7]. The chemical structure of the peak with retention time at 25.702 remained unclear. According to MS^2 data, it is supposed to be an unknown ester of **4**.

The most important fragmentation pathway of flavonoids is the RDA (retro Diels-Alder) cleavage. It occurs in six-member cyclic structures containing a double bond. During RDA reaction three pairs of electrons in the ring are relocated, resulting in the cleavage of two σ bonds and the formation of two π-bonds. Ma et al. proposed a nomenclature to unambiguously describe the resulting fragment ions of flavonids. In the negative ion mode the major ions are designated as ${}^{i,j}A^-$, containing the A ring, and ${}^{i,j}B^-$, containing the B-ring. The indices *i* and *j* represent the C-ring bonds that are broken [6, 8, 9].

C-ring cleavage product ions in RDA fragmentation can be used to determine the number and nature of the substituents on the A- and B-rings. In the MS/MS spectra of luteolin and apigenin, which have $[M - H]^-$ parent ions at $m/z \sim 284.9$ and ~ 268.9, respectively, an ${}^{1,3}A^-$ fragment ion appears at $m/z \sim 150.8$. The corresponding ${}^{1,3}B^-$ ions are found at $m/z \sim 116.8$ for apigenin, and at ~ 132.8 for **7**. This indicates that they differ in the substitution of the B-ring. Luteolin (**7**) has two OH groups, and apigenin one. 1,2 C-ring cleavage gives the main ion (${}^{1,2}A^-$) for quercetin (**6**) at $m/z \sim 178.8$. The complementary ion ${}^{1,2}B^-$ at $m/z \sim 120.8$ for **6** was observed in lower abundance. 0,4 C-ring cleavage gives the main ion (${}^{0,4}B^-$) for luteolin at $m/z \sim$ 174.8. ${}^{0,4}A^-$ and ${}^{1,3}B^-$ fragments are found in lower abundance. These fragments were observed for **7** (${}^{0,4}A^-$ at $m/z \sim 106.8$, ${}^{1,3}B^-$ at $m/z \sim 132.8$) and **6** (${}^{0,4}A^-$ at $m/z \sim 106.9$). 0,3 C-ring cleavage, generating ${}^{0,3}A^-$ and ${}^{0,3}B^-$ fragments, was not observed in this study. Besides various C-ring cleavages, other fragmentations were also observed, such as the loss of H_2O, CO, CO_2, successive loss of CO_2 and CO, and loss of the methyl radical ($\cdot CH_3$) [10, 11].

Phenolic compounds were characterized using the optimized LC/UV and LC/MS/MS analytical conditions. The HPLC-DAD method presented here proved to be reproducible, accurate, and sensitive for quantification of flavonol **6**, flavones **7**, **8**, **9**, and phenolic acids **1–5**. The LC/MS/MS technique provided structural information on flavonoids and phenolic acids based on various C-ring cleavages and loss of small fragments. Using tandem MS, besides the above-mentioned phenolic acids, vanillic acid, gallic acid, 5-*O*-feruloylquinic acid, and caffeic acid quinone were identified according to the literature data [4, 7].

EXPERIMENTAL

Plant Material. The aboveground parts of the plant *C. platycarpos* were collected in the surroundings of Imotski in June 2008 and air dried. The plant material was authenticated by Prof. Nikola Kujundzic, and voucher specimens were deposited in the Department of Analytical Chemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

Standards and Reagents. Apigenin, luteolin, quercetin, and butylated hydroxytoluene (BHT) were purchased from Fluka (Buchs, Switzerland). Chlorogenic acid (3-*O*-caffeoylquinic acid), caffeic acid, ferulic acid, isoferulic acid, and *o*-coumaric acid were from Acros Organics (Geel, Belgium). Chrysoeriol was from Extrasynthese (Genay, France). Methanol of HPLC grade was from Merck (Darmstadt, Germany). Ethyl acetate, chloroform, hydrochloric acid, and formic acid of analytical grade were purchased from Kemika (Zagreb, Croatia). Ammonium formate was of mass spectrometry grade from Fluka (Buchs, Switzerland). Water (0.055 μS/cm) was purified by a Milli-Q system from Millipore (Milford, USA).

Extraction and Hydrolysis of Plant Material. Air dried and ground, the aboveground parts of *C. platycarpos* were extracted as follows; about 1 g of accurately weighed plant material was extracted three times with 20 mL of methanol at 60°C

on a magnetic stirrer (3000 rpm) for 30 min. The filtered extracts were combined, and 60 mL of water was added. It was then extracted with chloroform to remove the chlorophyll. To 120 mL of the chlorophyll-free extract, 80 mL of acetone, 30 mL of 35% hydrochloric acid, and 345 mg of butylated hydroxytoluene (BHT) were added. The mixture, containing about 1.5 M HCl and 1.5 g/L BHT, was hydrolyzed by boiling on a water bath for 3 h protected from light. The hydrolyzed sample was purified by threefold liquid-liquid extraction with ethyl acetate saturated with water. Then the ethyl acetate extract was purified by extraction with water two times. The ethyl acetate phase was evaporated to dryness in vacuum at 40°C. The residue was taken up in 15.0 mL methanol and filtered through a 0.45 μm membrane filter.

Preparation of Standard Solutions. Standard stock solutions of five phenolic acids and four flavonoid aglycones were made in methanol at concentrations of 1.0 mg/mL and stored in a refrigerator at –20°C until use. All standard solutions were filtered through 0.45 μm filters. A working mixture of standards was subsequently prepared in methanol and diluted as necessary to provide a series of standards for constructing calibration curves for each of the target analytes.

Instrumentation (HPLC-DAD and MS Conditions) and Chromatographic Analyses. Analyses were performed on an Agilent 1100 chromatograph equipped with a diode array detector and mass detector in series. A ZORBAX SB-C18, 4.6 × 250 mm, particle size 5 μm with a suitable guard column, was employed for the separation. The binary mobile phase consisted of solvents A (10 mM ammonium formate in water–methanol 85:15, v/v, pH 4.0 adjusted with formic acid) and B (10 mM ammonium formate in water–methanol 15:85, v/v, pH 4.0 adjusted with formic acid). The gradient elution started with 10% B and changed to 80% B in 50 min, then reached 10% B in 10 min. After each run the chromatographic system was equilibrated with 10% B for 10 min. The flow rate was 1.0 mL/min and split out 200 μL/min to MS. The injection volume was 5 μL.

Spectral data for all peaks were recorded in the range 200–600 nm. HPLC-MS analysis was performed using the same separation conditions as for HPLC-DAD analysis. The mass detector was an ion trap spectrometer (Agilent LC/MSD Trap VL) equipped with an electrospray ionization interface and controlled by LCMSD software. All mass spectrometry data were recorded in the negative ion mode. The ionization conditions were adjusted at 325°C and 3.5 kV for capillary temperature and voltage, respectively. The nebulizer pressure was 35 psig, and the nitrogen flow rate was 11 L/min. The screening was performed in the full scan mode covering the range from *m/z* 50 up to 1000, in auto MS^n and manual MS^2 mode, to fragment the major ions obtained in the first step. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas with voltage cycles from 0.3 up to 2.0 V.

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