JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Variation of Polyphenols and Betaines in Aerial Parts of Young, Field-Grown *Amaranthus* Genotypes

Stine Krogh Steffensen,[†] Hans Albert Pedersen,[§] Rodrigo Labouriau,[#] Anne G. Mortensen,[§] Bente Laursen,[§] Rosa M. de Troiani,[⊗] Elke J. Noellemeyer,[⊗] Dagmar Janovska,[⊥] Helena Stavelikova,[⊥] Andreu Taberner,[△] Carsten Christophersen,[†] and Inge S. Fomsgaard[*,△]

[†]Department of Chemistry, Faculty of Science, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark

[§]Department of Agroecology, Faculty of Science and Technology, Aarhus University, Forsøgsvej 1, DK-4200 Slagelse, Denmark

[#]Department of Molecular Biology and Genetics, Faculty of Science and Technology, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark

[⊗]Facultad de Agronomía, Universidad Nacional de La Pampa, Coronel Gil 353 Casilla de Correo 300, Santa Rosa, La Pampa AR-LP 6300, Argentina

[⊥]Crop Research Institute, Drnovska 507, Prague 6−Ruzyne 16106, Czech Republic

[△]Hortofruticultura, Botanica y Jardineria, Universidad de Lleida, Rovira Roure 191, Lleida 25198, Spain

ABSTRACT: *Amaranthus hybridus* and *Amaranthus mantegazzianus* are commonly cultivated and the entire young fresh plants consumed as vegetables in regions of Africa and Asia. *A. hybridus* and *A. mantegazzianus* were cultivated at four sites in three climate regions of the world: Santa Rosa, Argentina; Lleida, Spain; and Prague and Olomouc, both in the Czech Republic. The contents of flavonoids (isoquercitrin, rutin, nicotiflorin), hydroxybenzoic acids (protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid), hydroxycinnamic acids (caffeic acid, *p*-coumaric acid, ferulic acid), hydroxycinnamyl amides (*N-trans*-feruloyltyramine, *N-trans*-feruloyl-4-*O*-methyldopamine), and betaines (glycinebetaine, trigonelline) were determined. The variation in phytochemical content due to species and cultivation site was analyzed utilizing the multivariate statistical methods of principal component analysis (PCA) and graphical model (GM). The Argentinean samples differed from the three other locations due to higher contents of most compounds. The samples from Spain and the Czech Republic differed from each other in the content of the negatively correlated metabolites trigonelline and the flavonoids. The two amaranth species were separated primarily by a higher content of trigonelline and the two hydroxycinnamyl amides in *A. mantegazzianus*. The GM showed that the quantities of the different analytes within each compound group were intercorrelated except in the case of the betaines. The betaines carried no information on each other that was not given through correlations with other compounds. The hydroxycinnamic acids were a key group of compounds in this analysis as they separated the other groups from each other (i.e., carried information on all of the other groups). This study showed the contents of polyphenols and betaines in the aerial parts of vegetable amaranth to be very dependent on growth conditions, but also revealed that some of the compounds (trigonelline and the two hydroxycinnamyl amides) may be useful as features of a taxonomic classification.

KEYWORDS: *Amaranthus hybridus*, *Amaranthus mantegazzianus*, cultivation experiment, polyphenol, flavonoid, phenolic acid, betaine, variation, principal component analysis (PCA), graphical model (GM), chemical analysis, secondary metabolites

INTRODUCTION

Vegetable amaranth has traditionally been consumed in many regions of Africa and Asia.[1−5] Analysis of the protein content and amino acid composition has shown amaranth leaves as well as grains to be excellent sources of protein and essential amino acids that could provide a valuable supplement to a low-protein diet.[1,6] Amaranth leaves have been found to contain high amounts of vitamins such as vitamins A, B, and C and minerals such as potassium, calcium, and magnesium.[2,6,7] It is well-established that amaranth leaf extracts possess high antioxidant activities,[2,4,5,8] but despite this the content and composition of polyphenols in *Amaranthus* plant material have only been sporadically investigated. Kalinova and Dadakova[9] performed an extensive cultivation experiment on *Amaranthus* but analyzed only the rutin and total quercetin content of the plant material. Pasko et al.[10] found gallic acid and rutin in sprouts of *Amaranthus cruentus*. Alvarez-Jubete et al.[11] found only protocatechuic acid in sprouts of amaranth and no flavonoids. Miean and Mohamed[12] searched for a range of flavonoids and, after hydrolysis, found quercetin in leaves of *Amaranthus gangeticus*. Stintzing et al.[13] identified three quercetin glycosides (among them rutin and isoquercitrin), a kaempferol glycoside, and several hydroxycinnamate derivatives of quinic acid from the stem bark of *Amaranthus spinosus*. Recently, the content of polyphenols was analyzed in leaves of *Amaranthus hypochondriacus*, *Amaranthus mantegazzianus*, and *A. cruentus* in conjunction with biotic and abiotic stresses in cooperation with our group.[14,15] The hydroxycinnamyl amide *N-trans*-feruloyl-4-*O*-methyldopamine was isolated from *A. gangeticus* by Islam et al.[16] Previously, in our own group six hydroxycinnamyl amides were identified in *A. hypochondriacus* and *A. mantegazzianus*.[17,18] Understanding the variations in the content

Received: July 29, 2011
Revised: October 17, 2011
Accepted: October 18, 2011
Published: October 18, 2011

dx.doi.org/10.1021/jf202969e | *J. Agric. Food Chem.* 2011, 59, 12073–12082

Table 1. Name, Retention Time, and Mass Pair for Identification of Quantified Compounds in the Young Aerial Parts of *Amaranthus hybridus* K593 and *Amaranthus mantegazzianus* Don Juan

trivial name	abbrev	alternative name	t_R (min)	Q1/Q3[a]
		Flavonoids		
rutin	Rut	quercetin-3-*O*-rutenoside[b]	12.4	609.5/300.7
isoquercitrin	IsoQ	quercetin-3-*O*-glucoside[b]	16.3	463.3/300.4
nicotiflorin	Nic	kaempferol-3-*O*-rutenoside[b]	17.3	593.5/284.9
		Hydroxybenzoic Acids		
4-hydroxybenzoic acid	4HBA	4-hydroxybenzoic acid	8.7	137.2/92.8
salicylic acid	Sal	2-hydroxybenzoic acid	15.3	137.2/92.8
protocatechuic acid	Pro	3,4-dihydroxybenzoic acid	5.7	153.1/109.0
vanillic acid	Van	4-hydroxy-3-methoxybenzoic acid	9.7	167.2/152.0
		Hydroxycinnamic Acids		
p-coumaric acid	Cou	*trans*-3-(4′-hydroxyphenyl)propenoic acid	15.7	163.2/119.2
caffeic acid	Caf	*trans*-3-(3′,4′-dihydroxyphenyl)propenoic acid	9.1	179.0/135.0
ferulic acid	Fer	*trans*-3-(4′-hydroxy-3′-methoxyphenyl)propenoic acid	18.1	193.0/149.0
		Hydroxycinnamyl Amides		
N-*trans*-caffeoyltyramine	CafTyr	*N*-(*trans*-3″-(3‴,4‴-dihydroxyphenyl)propenoyl)-2-(4′-hydroxyphenyl)ethylamine	6.6	298.0/135.2
N-*trans*-feruloyltyramine	FerTyr	*N*-(*trans*-3″-(4‴-hydroxy-3‴-methoxyphenyl)propenoyl)-2-(4′-hydroxyphenyl)ethylamine	9.1	312.1/148.0
N-*trans*-feruloyl-4-*O*-methyldopamine	Fer4O	*N*-(*trans*-3″-(4‴-hydroxy-3‴-methoxyphenyl)propenoyl)-2-(4′-hydroxy-3′-methoxyphenyl)ethylamine	10.7	342.2/190.1
		Betaines		
glycinebetaine	Bet	2-(trimethylammonium)acetate	3.74	118.5/58.3
trigonelline	Tri	1-methylpyridinium-3-carbonate	3.89	138.2/92.1

[a] The flavonoids, hydroxybenzoic acids, and hydroxycinnamic acids were analyzed by chromatographic method 1 run in negative ionization mode. The hydroxycinnamyl amides were analyzed by chromatographic method 3 run in negative ionization mode. The betaines were analyzed by chromatographic method 2 run in positive ionization mode. [b] Not IUPAC name.

of these compounds is important as they have impacts on both plant fitness and the nutritional properties of vegetable amaranth. The purpose of this study was therefore to investigate the variations in the content of polyphenols (flavonoids, hydroxybenzoic acids, hydroxycinnamic acids, hydroxycinnamyl amides) and simple betaines (glycinebetaine, trigonelline) in vegetable amaranth between four different cultivation sites in three different climate regions (Santa Rosa, Argentina; Lleida, Spain; Prague, Czech Republic; and Olomouc, Czech Republic) and between two species (*Amaranthus hybridus* K593 and *Amaranthus mantegazzianus* Don Juan). Principal component analysis (PCA) was applied to investigate how sample variance was affected by differences in cultivation site and genotype. The graphical model (GM) was applied to reveal actual correlations between the 13 different secondary metabolites analyzed while repressing spurious and redundant correlations. The *Amaranthus* genotypes *A. hybridus* K593 and *A. mantegazzianus* Don Juan were selected on the basis of their properties as vegetables and for their seed-producing abilities. These parameters are important for the farmer as they directly affect the appearance of the crop and the ability to generate seeds for next year's sowing. This study was part of the AMARANTH:FUTURE-FOOD project financed by the European Commission under the sixth framework program. The fields used for the cultivation trials belonged to the faculties of our agronomic partners in the project and represented climatically diverse regions where amaranth could be cultivated.

MATERIALS AND METHODS

Chemicals. All organic solvents used for extraction and analysis were of standard HPLC grade, and only Milli-Q grade water was used (Millipore Milli-Q lab water system, Billerica, MA). The following commercial standards were used for quantification: rutin, isoquercitrin, and trigonelline (Extrasynthèse, Genay, France); nicotiflorin (Carl-Roth GmbH, Karlsruhe, Germany); ferulic acid, *p*-coumaric acid, vanillic acid, and 4-hydroxybenzoic acid (Fluka, Brøndby, Denmark); and caffeic acid, protocatechuic acid, salicylic acid, and glycinebetaine (Sigma-Aldrich, Brøndby, Denmark). *N*-*trans*-Caffeoyltyramine, *N*-*trans*-feruloyltyramine, and *N*-*trans*-feruloyl-4-*O*-methyldopamine were synthesized in our laboratory.[18]

Plant Material and Sample Preparation. *Collection of Plant Material.* The vegetable amaranth genotype *A. hybridus* K593 was cultivated concurrently in Santa Rosa, Argentina; Lleida, Spain; and Prague and Olomouc, Czech Republic, in the growth season of 2008. The vegetable genotype *A. mantegazzianus* Don Juan was cultivated concurrently in Santa Rosa, Argentina, and Lleida, Spain. It was not possible to cultivate *A. mantegazzianus* in the Czech Republic as it was very sensitive to day length and would not mature under the conditions in the Czech experimental fields.[19] The cultivation trials were performed in a randomized block design with four blocks according to a standardized field plan previously designed to minimize the effects of local variation within the same field.[20] Three successive plants from the second internal row of the plot were sampled in each block. The young, aerial parts of the *Amaranthus* plants were harvested in the vegetative

growth stage when the plants had reached a height of 20−25 cm as is the practice in Asia, where the entire young plant is used and prepared as spinach.[21] The harvested plant material contained the entire aerial part of the plants. Only 2 cm was left above the ground. The freshly harvested plants were frozen at −20 °C. Once frozen, they were lyophilized for 48 days. The dry plant material was immediately shipped to Denmark by post.

Extraction of Samples for Chemical Analysis. Ground samples were extracted in duplicate using a Dionex ASE 350 accelerated solvent extraction (ASE) system. Ground sample (0.1 g) was placed between two layers of 5 g of chemically inert Ottawa sand in the 33 mL extraction cells. A filter was placed on top of the sand, and the extraction cell was filled with glass beads. The extraction solvent was 70% aqueous methanol (v/v), and the ASE extraction protocol used the following settings: preheat for 5 min, heat for 5 min, static for 3 min, flush 80%, purge for 60 s, 4 cycles, pressure, 1500 psi, and temperature, 80 °C. The extracts were stored in brown vials at −20 °C for a maximum of 2 months until chemical analysis. Different parameters for solvent and temperature of the extraction were applied for the hydroxycinnamyl amides (100% methanol; 120 °C).

Chemical Analysis by HPLC-MS/MS. *Equipment.* All analyses were performed on an Agilent 1200 HPLC coupled with an Applied Biosystems MDS Sciex API 3200 liquid chromatography−ion trap quadrupole mass spectrometer (LCMSMS) using electrospray ionization and multiple reaction monitoring (MRM). Before analysis, the extracts were diluted with an equal volume of water and filtered through a Sartorius SRP 15, 0.45 μm PTFE membrane filter. A flow of 0.2 mL min^{-1} and an injection volume of 20 μL were used.

Chromatographic Method 1. Negative-mode ionization was used for analysis of the hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids. A Synergi 4 μ Polar RP 80 A column from Phenomenex (2.0 × 250 mm) was equilibrated to a temperature of 30 °C. The chromatographic separation was obtained by a gradient of eluent A [7% aqueous acetonitrile (v/v), 20 mM glacial acetic acid] toward eluent B (78% aqueous acetonitrile, 20 mM glacial acetic acid) by the following cycle: 0−1 min, isocratic at 84% A; 1−5 min, gradient to 82% A; 5−22 min, gradient to 70% A; 22−30 min, gradient to 0% A; 30−35 min, isocratic at 0% A; 35−39 min, gradient to 84% A; 39−48 min, isocratic at 84% A.

Chromatographic Method 2. Positive-mode ionization was used for analysis of the betaines. A Synergi 4 μ Polar-RP 80 A column from Phenomenex (2.0 × 250 mm) was equilibrated to a temperature of 30 °C. The chromatographic separation was obtained by a gradient of eluent A [7% aqueous acetonitrile (v/v), 20 mM glacial acetic acid] toward eluent B (78% aqueous acetonitrile, 20 mM glacial acetic acid) by the following cycle: 0−4 min, isocratic at 100% A; 4−7 min, gradient to 84% A; 7−15 min, gradient to 70% A; 15−18 min, gradient to 0% A; 18−21 min, isocratic at 0% A; 21−23 min, gradient to 100% A; 23−35 min, isocratic at 100% A.

Chromatographic Method 3. Negative-mode ionization was used for analysis of the hydroxycinnamyl amides. A Synergi 4 μ Fusion-RP 80 A column from Phenomenex (2.0 × 250 mm) was equilibrated to a temperature of 30 °C. The chromatographic separation was obtained by a gradient of eluent A [10% aqueous acetonitrile (v/v), 20 mM glacial acetic acid] toward eluent B (100% acetonitrile, 20 mM glacial acetic acid) by the following cycle: 0−10 min, isocratic at 70% A; 10−21 min, gradient to 10% A; 21−23 min, isocratic at 10% A; 23−35 min, gradient to 70% A; 25−35 min, isocratic at 70% A.

Identification and Quantification. The presence of compounds in samples was verified by reference to MRM transitions and retention times of standards listed in Table 1. Quantification was carried out using calibration curves prepared using serial dilutions of stock solutions. The standard solutions were prepared in 50% methanol and 50% water (v/v) in the following concentrations: 3.13, 6.25, 12.5, 25.0, 50.0, 100, 200, 400, and 800 μg/L. The standard curves were fitted to a quadratic function with a weighting of $1/x$ because there were more data points at the lower end of the curve (correlation coefficient > 0.99).

Limit of Detection of the Analytical Method. According to EURACHEM,[22] the limit of detection (LoD) of an analytical method normally is determined as 3*s*, *s* being the sample standard deviation of 6−10 repeated measurements of (a) sample blank values or (b) fortified sample blank values and "3" stemming from the Student *t* distribution ($p = 0.01$). In our case sample blanks without the analytes did not exist. The sample standard deviation was therefore estimated from the difference of measurements of sets of duplicate extractions according to Taylor.[23] Only chemical determinations of ≤10 μg/g dry weight were used for the estimate.

Data Models and Model Validation. The quantification results were subjected to PCA using the program LatentiX 2.00 (Latent5, Frederiksberg, Denmark, 2005−2008). The data were scaled to unit variance before modeling. The model was based on the double determinations of 44 *Amaranthus* samples. Thirteen variables (concentrations of 13 secondary metabolites) were applied, and the model was inspected for clustering of samples corresponding to four category variables (cultivation site, species, repetition plot number, and analytical replicate number). Only one chemical determination of an analyte was equal to zero. As this presented no problem in the statistical modulation, no further action was taken to deal with it. The data set was investigated for outliers by the residual variance versus Hotelings plot and by visual inspection of the entire data set and smaller subsets. No samples were removed as outliers.

Graphical Model. The association between the 13 chemical determinations was studied using graphical models.[24−26] In this approach the chemical determinations are represented by random variables that form the vertices of a graph. A graph is a collection of vertices (graphically represented as points) and unordered pairs of vertices termed edges (graphically represented as lines joining the vertices). Two vertices are joined by an edge (line) whenever the conditional correlation between them, given all the other variables, is different from zero. According to the theory of GMs,[24] two variables are directly connected in the graph if, and only if, they carry some information on each other that is not already contained in the other variables present in the graph.

The GM representing the 13 chemical determinations was inferred by inverting the sample Pearson covariance matrix, which provides an estimate of the precision matrix (a matrix for which the *i*−*j*th entries give the conditional covariance between the *i*th and the *j*th variable given the other variables). Simultaneously, a nonparametric bootstrap test of equality to zero of each of the conditional covariances of the precision matrix was performed (10000 bootstrap repetitions). This procedure does not require multivariate normality of the data. Additionally, the *p* values of those tests were adjusted for multiple testing by the false discovery rate method.[27] The pairs of variables presenting conditional covariance statistically differing from zero at a 1% level of significance were included as edges in the GM. Graphical representations were produced using the R package gRapHD.[28]

RESULTS AND DISCUSSION

Limit of Detection of the Analytical Method. The limits of detection were as follows: isoquercitrin, 1.31 μg/g; protocatechuic acid, 3.67 μg/g; 4-hydroxybenzoic acid, 3.55 μg/g; vanillic acid, 3.56 μg/g; salicylic acid, 0.71 μg/g; *p*-coumaric acid, 1.97 μg/g; ferulic acid, 2.28 μg/g; *N*-*trans*-feruoyltyramine, 1.29 μg/g; *N*-*trans*-feruloyl-4-*O*-methyldopamine, 3.63 μg/g; *N*-*trans*-caffeoyltyramine, 0.08 μg/g. In all samples, rutin, nicotiflorin, caffeic acid, glycinebetaine, and trigonelline were found only in high concentrations (>10 μg/g dry weight), and the calculation of the

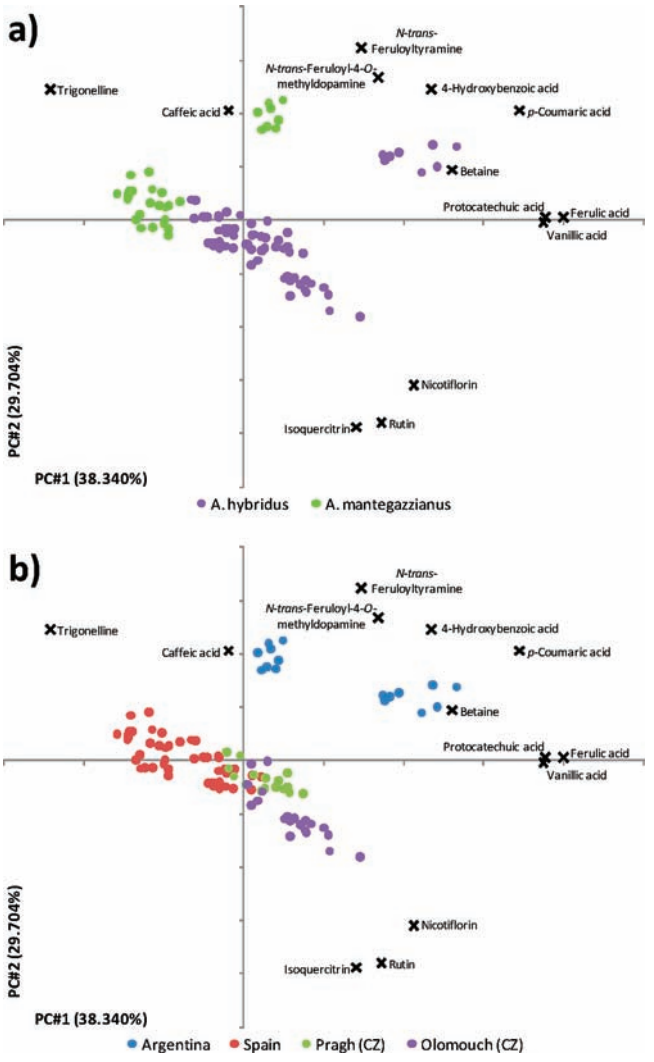

Figure 1. Principal component analysis biplot of PC1 versus PC2: (a) sample scores colored for genotype; (b) sample scores colored for cultivation site. The scores of the samples are denoted by small, colored spheres. The loadings of the variables are denoted by crosses and the names of the corresponding compounds.

LoD was not relevant for interpretation of the data set. Limits of detection of an analytical method, as calculated according to guidelines such as those of Eurachem[22] and with a significance level of $p = 0.01$, are of high importance for reporting and making decisions on important matters that depend on the certainty of the conclusions. Not all of the examined natural compounds in this paper were present above the LoD, but they are included nonetheless because quantification with higher uncertainties than the p value defined by Eurachem are possible, and the error in the overall conclusion would be higher if such numbers were omitted than if they were included in the multivariate statistical models.[29]

Multivariate Statistical Analysis. The 15 compounds listed in Table 1 was detected in the young aerial parts of the *Amaranthus* genotypes, *A. hybridus* K593 and *A. mantegazzianus* Don Juan, cultivated in the four different experimental locations. PCA was performed on data from the 13 compounds detected in >50% of the samples (isoquercitrin, rutin, nicotiflorin, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, *N-trans*-feruloyltyramine, *N-trans*-feruloyl- 4-*O*-methyldopamine, glycinebetaine, and trigonelline). Figure 1 displays the biplot of PC1 versus PC2 colored for (a) genotype and (b) cultivation site. The plot of the first two principal components explains 68.0% of the total variance and shows distinctive clustering of both the two genotypes and the four different experimental fields. No clustering was seen from block or plant number denoted by the field plan in the total sample set. In the PCA biplot variables with similar loadings appear to be correlated, as exemplified by the loadings of ferulic, vanillic, and protocatechuic acid in Figure 1. Those correlations could be spurious, however, because uncorrelated variables may have similar loadings. Moreover, an apparent correlation between two variables may appear merely because both variables are correlated to a third variable. We used the GM to systematically describe the interdependency between the 13 chemical determinations while avoiding redundant or spurious associations. Table 2 shows the sample Pearson correlations (upper right triangle) and the sample conditional correlation (lower left triangle) between each pair of variables formed with the 13 chemical determinations. Figure 2 displays the interdependence graph of the GM with the edges (lines in the graph) representing all of the statistically significant conditional correlations (at the 1% level) of the sample set. The gray edges connecting protocatechuic acid indicate that the conditional correlations for protocatechuic acid are less statistically significant ($p \leq 0.015$). The vertices are all connected in the graph, indicating a general interdependency between the chemical determinations.

Flavonoids. The flavonoids rutin, nicotiflorin, and isoquercitrin were quantified in the plant material, and their concentrations are shown in Figure 3. Rutin was the most abundant flavonoid in the aerial parts just as our previous papers have shown in the seeds.[30,31] As previously reported by Kalinova and Dadakova,[9] we saw that flavonoid concentrations were several times higher in the plant material than in the seeds,[31] and they showed lower relative variability between cultivation sites. Even so, large variations were seen, especially for rutin and isoquercitrin. In the young aerial parts as in the seeds,[31] nicotiflorin appears to be influenced less by environmental factors as the concentration varies little between the different cultivation sites. The GM of the data (see Figure 2 and Table 2) shows the rutin content in the plant material to be positively associated with both isoquercitrin and nicotiflorin. The lack of an edge between nicotiflorin and isoquercitrin indicates that the concentrations of these two substances do not carry information about each other that is not already given in the concentration of rutin. This is consistent with the biosynthetic pathway of nicotiflorin diverging from the biosynthetic pathway of the two other flavonoids.[32−35] The greater stability of nicotiflorin content to environmental factors indicates that nicotiflorin plays a different role in the aerial parts of *Amaranthus* than rutin and isoquercitrin. The 3′,4′-dihydroxy groups of the of quercetin B ring (the aglycone of both rutin and isoquercitrin) enable *o*-quinone formation as a very favorable mode of action.[36,37] It appears that *A. hybridus* in general has a higher content of reputed antioxidants, which should make *A. hybridus* the healthier choice.

In the PCA biplot of the first two components (see Figure 1) an axis can be drawn from the flavonoids through to trigonelline. Along this line *A. mantegazzianus* is separated from *A. hybridus* by higher concentrations of the flavonoids in the latter. The samples from Lleida, Prague, and Olomouc are likewise separated from each other along this line. This pattern indicates a correlation

Table 2. Pairwise (Pearson) Correlations (Upper Triangle) and Conditional Correlations (Lower Triangle) between the 13 Chemical Determinations[a]

	IsoQ	Nic	Rut	Pro	4HBA	Van	Caf	Cou	Fer	FerTyr	Fer4O	Bet	Tri
IsoQ	1.00	0.77 (<0.001)	0.87 (<0.001)	0.23 (0.017)	-0.25 (0.003)	0.31 (0.001)	-0.40 (<0.001)	-0.11 (0.137)	0.26 (0.010)	-0.37 (<0.001)	-0.31 (0.001)	-0.10 (0.137)	-0.68 (<0.001)
Nic	-0.30 (0.029)	1.00	0.87 (<0.001)	0.44 (<0.001)	-0.12 (0.077)	0.36 (<0.001)	-0.26 (0.001)	0.12 (0.049)	0.45 (<0.001)	-0.25 (0.004)	-0.09 (0.176)	0.12 (0.044)	-0.45 (<0.001)
Rut	**-0.50** (<0.001)	**-0.52** (0.001)	1.00	0.39 (<0.001)	-0.34 (<0.001)	0.28 (<0.001)	-0.45 (<0.001)	-0.04 (0.322)	0.37 (<0.001)	-0.36 (0.002)	-0.18 (0.069)	0.06 (0.240)	-0.56 (<0.001)
Pro	0.11 (0.247)	-0.09 (0.247)	0.01 (0.449)	1.00	0.35 (0.017)	0.72 (<0.001)	-0.01 (0.458)	0.72 (<0.001)	0.88 (<0.001)	0.31 (<0.001)	0.42 (<0.001)	0.54 (<0.001)	-0.43 (<0.001)
4HBA	0.18 (0.189)	**-0.29** (0.006)	0.25 (0.021)	0.35 (0.013)	1.00	0.65 (<0.001)	0.36 (0.002)	0.71 (<0.001)	0.53 (<0.001)	0.45 (<0.001)	0.27 (0.025)	0.33 (0.003)	-0.13 (0.112)
Van	**-0.38** (0.009)	0.10 (0.244)	0.17 (0.151)	-0.24 (0.071)	**-0.63** (<0.001)	1.00	-0.13 (0.150)	0.66 (<0.001)	0.81 (<0.001)	0.24 (0.001)	0.19 (0.019)	0.57 (<0.001)	-0.55 (<0.001)
Caf	-0.32 (0.145)	-0.08 (0.308)	**0.51** (0.006)	0.04 (0.386)	-0.21 (0.086)	**0.51** (<0.001)	1.00	0.31 (0.014)	0.16 (0.137)	0.00 (0.473)	-0.04 (0.326)	-0.13 (0.139)	0.19 (0.022)
Cou	0.23 (0.062)	-0.14 (0.220)	-0.02 (0.440)	0.05 (0.446)	-0.18 (0.172)	0.09 (0.198)	-0.18 (0.076)	1.00	0.82 (<0.001)	0.60 (<0.001)	0.55 (<0.001)	0.46 (<0.001)	-0.25 (0.005)
Fer	0.18 (0.219)	0.06 (0.377)	**-0.38** (0.006)	-0.48 (0.015)	-0.02 (0.478)	**-0.36** (0.001)	**-0.46** (<0.001)	**-0.52** (<0.001)	1.00	0.21 (0.002)	0.29 (<0.001)	0.56 (<0.001)	-0.56 (<0.001)
FerTyr	-0.36 (0.018)	0.16 (0.188)	0.24 (0.029)	-0.06 (0.386)	-0.13 (0.189)	-0.06 (0.290)	**0.31** (0.007)	**-0.52** (<0.001)	0.20 (0.076)	1.00	0.88 (<0.001)	0.26 (<0.001)	0.27 (0.008)
Fer4O	0.17 (0.129)	-0.09 (0.260)	-0.11 (0.200)	-0.13 (0.180)	0.07 (0.247)	0.23 (0.029)	0.00 (0.475)	0.14 (0.174)	-0.13 (0.162)	**-0.78** (<0.001)	1.00	0.35 (<0.001)	0.23 (0.021)
Bet	0.25 (0.038)	-0.17 (0.136)	0.16 (0.179)	0.14 (0.164)	0.20 (0.157)	-0.28 (0.076)	0.23 (0.086)	0.13 (0.151)	**-0.29** (0.008)	0.12 (0.179)	-0.19 (0.097)	1.00	-0.16 (0.048)
Tri	**0.64** (<0.001)	**-0.35** (0.008)	-0.21 (0.076)	-0.06 (0.308)	-0.09 (0.247)	-0.14 (0.161)	**0.37** (0.001)	-0.19 (0.103)	-0.31 (0.057)	**-0.34** (0.006)	0.07 (0.260)	-0.02 (0.438)	1.00

[a] The abbreviations can be found in Table 1. The *p* values of nonparametric bootstrap tests for equality to zero of each of the correlations or conditional correlations are given in parentheses; the *p* values are based on 10000 bootstrap repetitions and were adjusted for multiple testing by the false discovery rate method; statistically significant conditional correlations at a 1% level were entered as vertices in the graphical models and are displayed in bold and underlined and significance at 1.5% underlined.

between the flavonoids and trigonelline. In the GM (see Figure 2 and Table 2) the correlation is depicted in greater detail. Isoquercitrin and nicotiflorin are directly correlated to trigonelline (indicated by an edge between the vertices in Figure 2), but rutin is only indirectly correlated through its correlation to the two other flavonoids. In the GM (Figure 2) it is shown that rutin is correlated only to caffeic and ferulic acid apart from the other flavonoids. Rutin, caffeic acid, and ferulic acid are biosynthetically connected through the phenylpropanoid pathway and the common precursor *p*-coumaric acid,[32,38] but in our data we observed that rutin was not directly correlated with *p*-coumaric acid. Several factors play a role in the regulation of different parts of this pathway.[39] A connection between rutin and caffeic acid due to their structural similarity and antioxidant activity has been reported earlier.[37,40] Figures 1, 3, and 4 illustrate that the observed correlation between rutin and caffeic acid (Pearson = −0.45, $p < 0.001$; conditional = 0.51, $p = 0.006$) results from regional differences and not differences between the two *Amaranthus* species. Burchard et al.[41] reported hydroxycinnamic acid conjugates to be the most important UV-protectant compounds in young rye leaves, whereas the importance of the flavonoids increased with age to be the foremost important UV-protectant in mature rye leaves. However, both flavonoids and hydroxycinnamic acids were induced by UV radiation.[41]

Hydroxycinnamic Acids. Three hydroxycinnamic acids, caffeic acid, *p*-coumaric acid, and ferulic acid, were quantified in the leaf samples (see Figure 4). In the PCA plot of Figure 1 the loadings of caffeic, *p*-coumaric, and ferulic acid are spread over the plot area. The loading of ferulic acid is high in the first PC and is important for separating both the genotypes and the

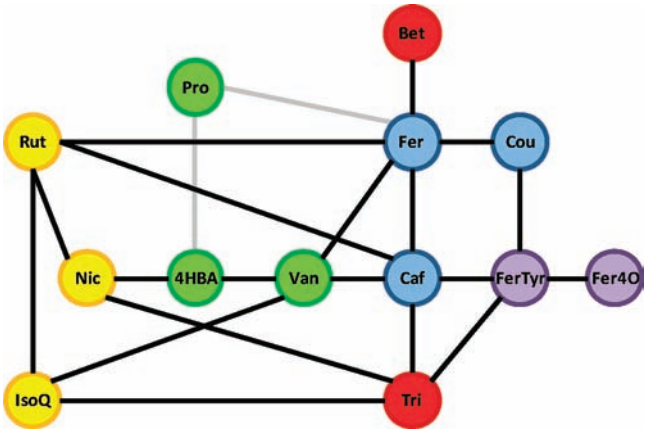

Figure 2. Graphical model (GM) representing the conditional correlations between the analytes (see the lower triangle of Table 2). The abbreviations can be seen in Table 1. The vertices (colored spheres) represent the 13 different analytes: yellow, flavonoids; green, hydroxybenzoic acids; blue, hydroxycinnamic acids; purple, hydroxycinnamyl amides; red, betaines. The edges (lines joining the vertices) represent conditional correlation between the content of the connected analytes (black edges, $p \leq 0.010$; gray edges, $p \leq 0.015$). The absence of an edge between two vertices indicates that the conditional correlation between them is not statistically significant ($p \geq 0.015$). A conditional correlation does not proceed through a nodal point. The graph is unweighted in the sense that there is no meaning attributed to the length of the edges in the graphical representations.

cultivation sites. Caffeic acid separates the Spanish and Argentinean samples from both Czech sample sets along PC2, the first

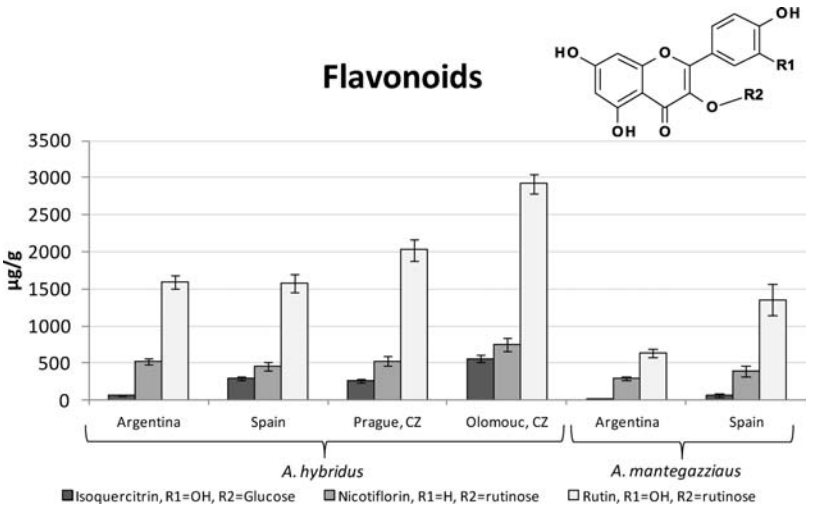

Figure 3. Content of flavonoids in *Amaranthus* plant material (μg/g dry weight). The error bars in the columns indicate the combined standard deviations of field replicates and chemical analysis.

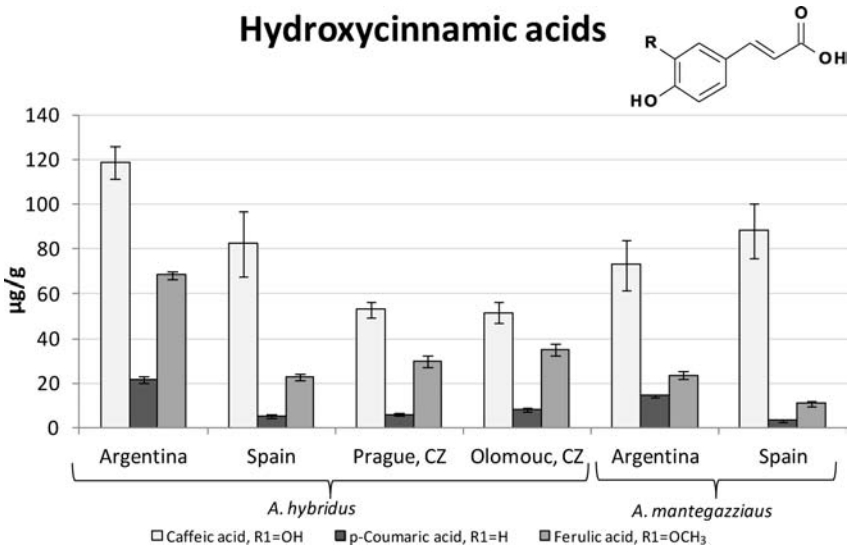

Figure 4. Content of the hydroxycinnamic acids in *Amaranthus* plant material (μg/g dry weight). The error bars in the columns indicate the combined standard deviations of field replicates and chemical analysis.

having a higher content than the latter. A higher content of *p*-coumaric acid in the Argentinean samples significantly separates them from the rest. The orthogonality of the loadings of ferulic acid and caffeic acid in the PCA suggests that the concentrations of the two are regulated independently. However, the conditional correlation matrix (see Table 2) reveals that when all of the other correlations in the data set are taken into account, ferulic acid and caffeic acid are shown to be strongly, negatively correlated (-0.46, $p < 0.001$). This is depicted graphically by an edge between the two vertices in Figure 2. *p*-Coumaric acid also exhibits a strong correlation to ferulic acid (Pearson correlation = 0.82, $p < 0.001$; conditional correlation = -0.52, $p < 0.001$). In the phenylpropanoid biosynthetic pathway *p*-coumaric acid is the precursor for caffeic acid, which is in turn the precursor for ferulic acid.[38] The correlation pattern seen in our data suggests that the regulation of the synthesis is not straightforward. *p*-Coumaric acid is the least abundant hydroxycinnamic acid in the aerial parts. In the seeds, however, though not detectable in high concentrations, *p*-coumaric acid was the only hydroxycinnamic acid detectable in >50% of amaranth seed samples.[31]

In the GM (see Figure 2) it is important to note the hydroxycinnamic acids (together with trigonelline) separate the flavonoids and hydroxybenzoic acids from the hydroxycinnamyl amides and from glycinebetaine. This means that each of these isolated groups brings no new information to any of the other isolated groups that is not already given by the correlation to the *isolating* group (the hydroxycinnamic acids). For instance, the content of flavonoids gives no information on the content of hydroxycinnamyl amides that is not already given by the content of the hydroxycinnamic acids and trigonelline. It follows that if the compounds of main interest were the hydroxycinnamyl amides, it would not be necessary to analyze the flavonoids and hydroxybenzoic acids as the information they could give was already contained in the hydroxycinnamic acids and trigonelline.

Hydroxybenzoic Acids. Four hydroxybenzoic acids were detected in the aerial parts of vegetable amaranth: protocatechuic

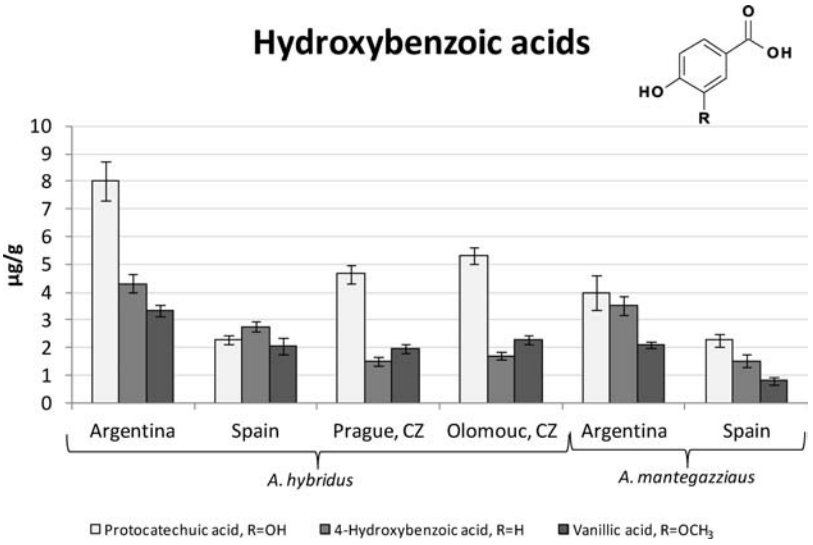

Figure 5. Content of the hydroxybenzoic acids in *Amaranthus* plant material (μg/g dry weight). The error bars in the columns indicate the combined standard deviations of field replicates and chemical analysis.

acid, 4-hydroxybenzoic acid, vanillic acid, and salicylic acid. The concentrations of the former three are given in Figure 5. Protocatechuic acid is the most abundant hydroxybenzoic acid in amaranth plant material, but the concentration varies substantially among the cultivation sites. These variations are less pronounced than for amaranth seeds, where similar concentrations were found.[31] 4-Hydroxybenzoic acid content varied between cultivation sites, whereas vanillic acid content was influenced only slightly by the change of environment. In the PCA plot (see Figure 1) the loadings of protocatechuic acid and vanillic acid are placed in close proximity on PC1. The clustering of the loadings in the PCA indicates a correlation. The GM (see Figure 2) shows that the hydroxybenzoic acid derivatives in general are closely correlated (displayed in green) and both protocatechuic and vanillic acid are directly, conditionally correlated to the hydroxycinnamic acid ferulic acid at a significance level of 1.5%. However, protocatechuic acid and vanillic acid are not directly, conditionally correlated. The group of hydroxybenzoic acids does not act to separate any other two groups in the GM from each other (other edges interconnect the other groups around the hydroxybenzoic acid derivatives). This indicates that the abundance of the chemicals of the other groups act independently of the abundance of the hydroxybenzoic acid derivatives as can be deduced using basic theory of GMs.[24]

Salicylic acid was found in most samples in concentrations comparable to the LoD, but some individual plants displayed abnormal concentrations as high as 9.58 μg/g. No patterns could be recognized in these spikes and, when included in the PCA model, produced dramatic outliers. The quantification data on salicylic acid were therefore omitted from the statistical analyses. Salicylic acid is known to be an important signaling compound in plant defense response, for which reason the plants containing abnormally large concentrations likely represent plants recently attacked by a pathogen.[42] However, none of the other compounds quantified showed any correlation to the spikes of salicylic acid. Likewise, the content of salicylic acid has been shown to be uncorrelated to the concentration of the hydroxycinnamyl amides in tomatoes.[43]

Hydroxycinnamyl Amides. The aerial parts of the young amaranth plants were analyzed for the hydroxycinnamyl amides *N-trans*-caffeoyltyramine, *N-trans*-feruloyltyramine, and *N-trans*-feruloyl-4-*O*-methyldopamine. The latter two were detected in all samples, and the quantification data are presented in Figure 6. *N-trans*-Caffeoyltyramine was detected only in the Argentinean samples in very low concentrations, and consequently these data have been omitted from any further statistical analysis. The two hydroxycinnamyl amides investigated are strongly correlated (0.88, $p < 0.001$). Figures 1 and 6 demonstrate how the hydroxycinnamyl amides are important in distinguishing the Argentinean samples, which have higher contents of these compounds. Separate PCA plots of the Argentinean and Spanish sample sets reveal that the hydroxycinnamyl amides, together with trigonelline, are also important in separating *A. hybridus* from *A. mantegazzianus* (plot not shown). Hydroxycinnamyl amides have previously been implicated in mechanisms of defense against pathogenic attack and physical wounding in tobacco leaves.[44] Guillet et al.[44] demonstrated that not only was the production of these compounds induced by pathogenic attack but so was the production of enzymes responsible for hydroxycinnamyl amide synthesis. It remains to be determined whether *A. mantegazzianus* has a higher content because it has a stronger response to pathogenic attacks or because it is more susceptible. However, in an investigation of the effect of herbivore on polyphenols of different *Amaranthus* genotypes, *A. mantegazzianus* was shown to be significantly less attacked by herbivores than the other investigated genotypes.[15] Plants for this trial were cultivated concurrently with those used in the present study and on the same experimental fields in Santa Rosa, Argentina. The high content of these compounds in samples grown in Argentina over samples grown in Europe indicates that the Argentinean plants were under much greater pressure from pathogens than European amaranth plants.

The GM (see Figure 2) shows that all information on the *N-trans*-feruloyl-4-*O*-methyldopamine content from the rest of the variables is contained in the content of *N-trans*-feruloyltyramine. *N-trans*-Feruloyltyramine, on the other hand, is strongly correlated to *p*-coumaric acid (0.82, $p < 0.001$). Figure 2 reveals an association between *N-trans*-feruloyltyramine and caffeic acid, but we found no association between *N-trans*-feruloyltyramine and ferulic acid.

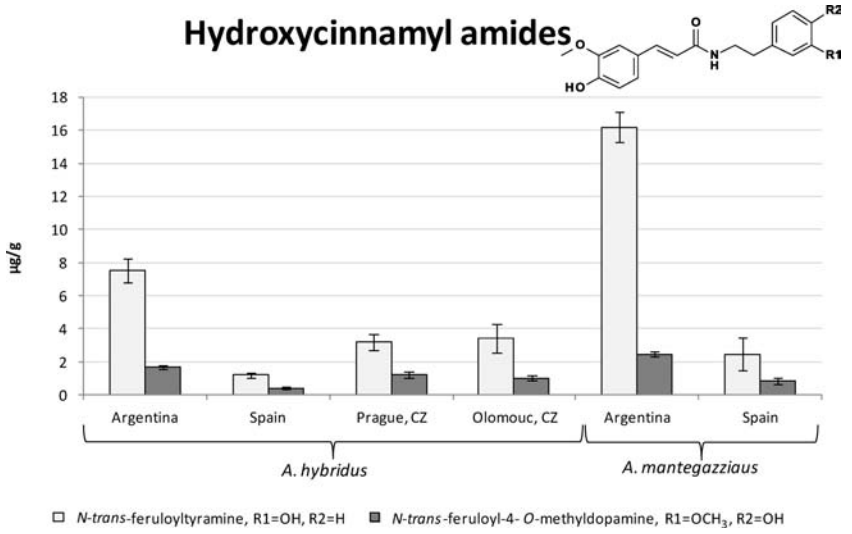

Figure 6. Content of hydroxycinnamyl amides in *Amaranthus* plant material (μg/g dry weight). The error bars in the columns indicate the combined standard deviations of field replicates and chemical analysis.

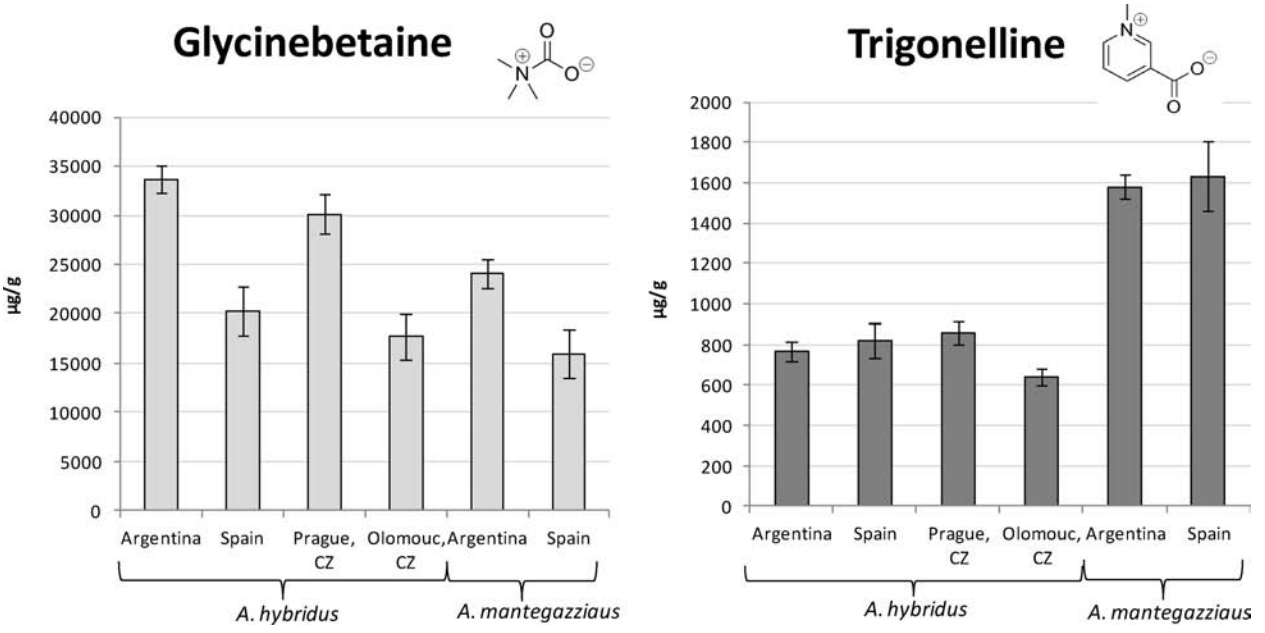

Figure 7. Content of betaines in *Amaranthus* plant material (μg/g dry weight). The error bars in the columns indicate the combined standard deviations of field replicates and chemical analysis.

This is surprising as it has been reported that ferulic acid and its coenzyme A analogue are precursors for the ferulyl amides.[45] The GM shows a significant conditional correlation between *N-trans*-feruloyltyramine and trigonelline. These compounds have neither structural similarity nor any reported common mode of action. However, this correlation might well be explained by their importance in distinguishing the two species in our study (see Figure 2).

Betaines. *Amaranthus* is known for its ability to accumulate betaines, especially glycinebetaine and trigonelline.[46] The betaines are known to counteract osmotic stress and are known to play an important role in salt and drought tolerance.[47] The concentrations of glycinebetaine and trigonelline found in the young aerial parts of *Amaranthus* plants are shown in Figure 7. In the PCA plot (see Figure 1) the loadings of glycinebetaine and trigonelline indicate that, despite their common role as osmoprotectants,[47] they are regulated independently. Glycinebetaine and trigonelline presented a marginally significant negative Pearson correlation (-0.16, $p = 0.048$) (see Table 2). On the other hand, glycinebetaine is highly correlated with ferulic acid (0.56, $p < 0.001$) and trigonelline is highly negatively correlated with ferulic acid (-0.56, $p < 0.001$). However, the conditional correlation between trigonelline and glycinebetaine, given ferulic acid, is only -0.02 and is not statistically significant ($p = 0.438$) (see Table 2). This indicates that the correlation between trigonelline and glycinebetaine is completely explained by the correlation of those two variables to a third variable (ferulic acid). This is corroborated by the work of Kim et al.,[48] who found that the biosynthesis of both glycinebetaine and lignin precursors appeared to be induced simultaneously

as a response to short-term salt stress in an *Arabibopsis* cell culture.

AUTHOR INFORMATION

Corresponding Author

*Phone: +45 89993610. Fax: +45 89993501. E-mail: inge.fomsgaard@agrsci.dk.

REFERENCES

(1) Abu Ziada, M. E.; El-Halawany, E. F.; Mashaly, I. A.; Masoud, G. F. Autecology and phytochemistry of genus *Amaranthus* in the Nile Delta, Egypt. *Asian J. Plant Sci.* **2008**, *7* (2), 119–129.

(2) Adefegha, S. A.; Oboh, G. Cooking enhances the antioxidant properties of some tropical green leafy vegetables. *Afr. J. Biotechnol.* **2011**, *10* (4), 632–639.

(3) Ghaly, A. E.; Alkoaik, F. N. Extraction of protein from common plant leaves for use as human food. *Am. J. Appl. Sci.* **2010**, *7* (3), 331–342.

(4) Gupta, S.; Prakash, J. Studies on Indian green leafy vegetables for their antioxidant activity. *Plant Foods Hum. Nutr.* **2009**, *64* (1), 39–45.

(5) Ozsoy, N.; Yilmaz, T.; Kurt, O.; Can, A.; Yanardag, R. In vitro antioxidant activity of *Amaranthus lividus* L. *Food Chem.* **2009**, *116* (4), 867–872.

(6) Fasuyi, A. O.; Akindahunsi, A. O. Nutritional evaluation of *Amaranthus cruentus* leaf meal based broiler diets supplemented with cellulase/glucanase/xylanase enzymes. *Am. J. Food Technol.* **2009**, *4* (3), 108–118.

(7) Akubugwo, I. E.; Obasi, N. A.; Chinyere, G. C.; Ugbogu, A. E. Nutritional and chemical value of *Amaranthus hybridus* L. leaves from Afikpo, Nigeria. *Afr. J. Biotechnol.* **2007**, *6* (24), 2833–2839.

(8) Gupta, S.; Prakash, J. Influence of antioxidant components on antioxidant activity of dehydrated green leafy vegetables. *Food Sci. Technol. Res.* **2008**, *14* (1), 104–109.

(9) Kalinova, J.; Dadakova, E. Rutin and total quercetin content in amaranth (*Amaranthus* spp.). *Plant Foods Hum. Nutr.* **2009**, *64* (1), 68–74.

(10) Pasko, P.; Sajewicz, M.; Gorinstein, S.; Zachwieja, Z. Analysis of selected phenolic acids and flavonoids in *Amaranthus cruentus* and *Chenopodium quinoa* seeds and sprouts by HPLC. *Acta Chromatogr.* **2008**, *20* (4), 661–672.

(11) Alvarez-Jubete, L.; Wijngaard, H.; Arendt, E. K.; Gallagher, E. Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. *Food Chem.* **2010**, *119* (2), 770–778.

(12) Miean, K. H.; Mohamed, S. Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *J. Agric. Food Chem.* **2001**, *49* (6), 3106–3112.

(13) Stintzing, F. C.; Kammerer, D.; Schieber, A.; Adama, H.; Nacoulma, O. G.; Carle, R. Betacyanins and phenolic compounds from *Amaranthus spinosus* L. and *Boerhavia erecta* L. *Z. Naturforsch. C* **2004**, *59* (1/2), 1–8.

(14) Barba de la Rosa, A. P.; Rodríguez-Enríquez, R. J.; Mortensen, A. G.; Laursen, B.; De León-Rodríguez, A.; Fomsgaard, I. S. Influence of growing conditions on the flavonoids and phenolic acid composition in amaranth (*Amaranthus hypochondriacus* L.) leaves. Unpublished work, 2011.

(15) Niveyro, S.; Mortensen, A. G.; Fomsgaard, I. S.; Salvo, A. Secondary metabolites of amaranth varieties (*Amaranthus* spp.) and its relationship with leaf damage by chewing insects in the field. Unpublished work, 2011.

(16) Islam, T.; Hashidoko, Y.; Ito, T.; Tahara, S. Interruption of the homing events of phytopathogenic *Aphanomyces cochlioides* zoospores by secondary metabolites from nonhost *Amaranthus gangeticus*. *J. Pestic. Sci.* **2004**, *29* (1), 6–14.

(17) Pedersen, H. A.; Steffensen, S. K.; Christophersen, C. Cinnamoylphenethylamine ^{1}H-NMR chemical shifts: a concise reference for ubiquitous compounds. *Nat. Prod. Commun.* **2010**, *5* (8), 1259–1262.

(18) Pedersen, H. A.; Steffensen, S. K.; Christophersen, C.; Mortensen, A. G.; Jørgensen, L. N.; Niveyro, S.; de Troiani, R. M.; Rodríguez-Enríquez, R. J.; Barba de la Rosa, A. P.; Fomsgaard, I. S. Synthesis and quantitation of six phenolic amides in *Amaranthus* spp. *J. Agric. Food Chem.* **2010**, *58* (10), 6306–6311.

(19) National Research Council. *Amaranth: Modern Prospects for an Ancient Crop*; National Academy Press: Washington, DC, 1984.

(20) Fomsgaard, I. S.; Añon, M. C.; Barba de la Rosa, A. P.; Christophersen, C.; Dusek, K.; Délano-Frier, J.; Espinoza Pérez, J.; Fonseca, A.; Janovská, D.; Kudsk, P.; Labouriau, R. S.; Lacayo Romero, M. L.; Martínez, N.; Matusová, K.; Mathiassen, S. K.; Noellemeyer, E. J.; Pedersen, H. A.; Stavalikova, H.; Steffensen, S. K.; de Troiani, R. M.; Taberner, A. *Adding Value to Holy Grain: Providing the Key Tools for the Exploitation of Amaranth — the Protein-Rich Grain of the Aztecs*; results from a joint European−Latin American research project;Department of Integrated Pest Management, Aarhus University, Faculty of Agricultural Science: Aarhus, Denmark, 2010.

(21) Jarošová, J.; Michalová, A.; Vavreinová, S.; Moudrý, J. *Cultivation and Utilization of Amaranth*; UZPI Praha: 1997.

(22) EURACHEM. *The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. EURACHEM Guide*, 1st English ed.; LGC: Teddington, U.K., 1998; http://www.eurachem.org/guides/pdf/valid.pdf

(23) Taylor, J. K. *Quality Assurance of Chemical Measurements*; Lewis Publishers: Boca Raton, FL, 1987.

(24) Whittaker, J. *Graphical Models in Applied Multivariate Statistics*; Wiley: Chichester, U.K., 1990.

(25) Labouriau, R.; Amorim, A. N. Comment on "An association between the kinship and fertility of human couples". *Science* **2008**, *322* (5908), 1634.

(26) Holmstrup, M.; Sørensen, J. G.; Overgaard, J.; Bayley, M.; Bindesbøl, A. M.; Slotsbo, S.; Fisker, K. V.; Maraldo, K.; Waagner, D.; Labouriau, R.; Asmund, G. Body metal concentrations and glycogen reserves in earthworms (*Dendrobaena octaedra*) from contaminated and uncontaminated forest soil. *Environ. Pollut.* **2011**, *159* (1), 190–197.

(27) Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* **1995**, *57*, 289–300.

(28) Abreu, G. C. G.; Edwards, D.; Labouriau, R. High-dimensional graphical model search with the gRapHD R package. *J. Stat. Softw.* **2010**, *37* (1), 1–18.

(29) Ellison, S. L. R.; Barwick, V. J.; Williams, A. Legislative limits below detection capability. *Accredit. Qual. Assur.* **2000**, *5* (8), 308–313.

(30) Barba de la Rosa, A. P.; Fomsgaard, I. S.; Laursen, B.; Mortensen, A. G.; Olivera-Martínez, L.; Silva-Sánchez, C.; Mendoza-Herrera, A.; González-Castañeda, J.; León-Rodríguez, A. Amaranth (*Amaranthus hypochondriacus*) as an alternative crop for sustainable food production: phenolic acids and flavonoids with potential impact on its nutraceutical quality. *J. Cereal Sci.* **2009**, *49* (1), 117–121.

(31) Steffensen, S. K.; Rinnan, A.; Mortensen, A. G.; Laursen, B.; de Troiani, R. M.; Noellemeyer, E. J.; Janovska, D.; Dusek, K.; Délano-Frier, J.; Taberner, A.; Christophersen, C.; Fomsgaard, I. S. Variations in the polyphenol content of seeds of field grown *Amaranthus* genotypes. *Food Chem.* **2011**, *129* (1), 131–138.

(32) Calvenzani, V.; Martinelli, M.; Lazzeri, V.; Giuntini, D.; Dall'Asta, C.; Galaverna, G.; Tonelli, C.; Ranieri, A.; Petroni, K. Response of wild-type and *high pigment-1*; tomato fruit to UV-B depletion: flavonoid profiling and gene expression. *Planta* **2010**, *231* (3), 755–765.

(33) Carvalho, I. S.; Cavaco, T.; Carvalho, L. M.; Duque, P. Effect of photoperiod on flavonoid pathway activity in sweet potato (*Ipomoea batatas* (L.) Lam.) leaves. *Food Chem.* **2010**, *118* (2), 384–390.

(34) Gautier, H.; Diakou-Verdin, V.; Benard, C.; Reich, M.; Buret, M.; Bourgaud, F.; Poessel, J. L.; Caris-Veyrat, C.; Genard, M. How does tomato quality (sugar, acid, and nutritional quality) vary with ripening stage, temperature, and irradiance? *J. Agric. Food Chem.* **2008**, *56* (4), 1241–1250.

(35) Suzuki, T.; Honda, Y.; Mukasa, Y. Effects of UV-B radiation, cold and desiccation stress on rutin concentration and rutin glucosidase

activity in tartary buckwheat (*Fagopyrum tataricum*) leaves. *Plant Sci.* **2005**, *168* (5), 1303–1307.

(36) Olsen, R. A.; Bennett, J. H.; Blume, D.; Brown, J. C. Chemical aspects of the Fe stress response mechanism in tomatoes. *J. Plant Nutr.* **1981**, 3 (6), 905–921.

(37) Zhang, J.; Melton, L.; Adaim, A.; Skinner, M. Cytoprotective effects of polyphenolics on H_2O_2-induced cell death in SH-SY5Y cells in relation to their antioxidant activities. *Eur. Food Res. Technol.* **2008**, *228* (1), 123–131.

(38) Schoch, G.; Goepfert, S.; Morant, M.; Hehn, A.; Meyer, D.; Ullmann, P.; Werck-Reichhart, D. CYP98A3 from *Arabidopsis thaliana* is a 3′-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. *J. Biol. Chem.* **2001**, *276* (39), 36566–36574.

(39) Tattini, M.; Galardi, C.; Pinelli, P.; Massai, R.; Remorini, D.; Agati, G. Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. *New Phytol.* **2004**, *163*, 547–561.

(40) Cetkovic, G. S.; Djilas, S. M.; Canadanovic-Brunet, J. M.; Tumbas, V. T. Antioxidant properties of marigold extracts. *Food Res. Int.* **2004**, *37* (7), 643–650.

(41) Burchard, P.; Bilger, W.; Weissenböck, G. Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultraviolet-induced chlorophyll fluorescence measurements. *Plant Cell Environ.* **2000**, *23* (12), 1373–1380.

(42) Hayat, Q.; Hayat, S.; Irfan, M.; Ahmad, A. Effect of exogenous salicylic acid under changing environment: a review. *Environ. Exp. Bot.* **2010**, *68* (1), 14–25.

(43) Zacares, L.; Lopez-Gresa, M. P.; Fayos, J.; Primo, J.; Belles, J. M.; Conejero, V. Induction of *p*-coumaroyldopamine and feruloyldopamine, two novel metabolites, in tomato by the bacterial pathogen *Pseudomonas syringae*. *Mol. Plant−Microbe Interact.* **2007**, *20* (11), 1439–1448.

(44) Guillet, G.; De Luca, V. Wound-Inducible biosynthesis of phytoalexin hydroxycinnamic acid amides of tyramine in tryptophan and tyrosine decarboxylase transgenic tobacco lines. *Plant Physiol.* **2005**, *137* (2), 692–699.

(45) Ishihara, A.; Kawata, N.; Matsukawa, T.; Iwamura, H. Induction of *N*-hydroxycinnamoyltyramine synthesis and tyramine *N*-hydroxycinnamoyltransferase (THT) activity by wounding in maize leaves. *Biosci., Biotechnol., Biochem.* **2000**, *64* (5), 1025–1031.

(46) Blunden, G.; Yang, M. h.; Janicsák, G.; Máthé, I.; Carabot-Cuervo, A. Betaine distribution in the Amaranthaceae. *Biochem. Syst. Ecol.* **1999**, *27* (1), 87–92.

(47) Oufir, M.; Schulz, N.; Sha Vallikhan, P. S.; Wilhelm, E.; Burg, K.; Hausman, J. F.; Hoffmann, L.; Guignard, C. Simultaneous measurement of proline and related compounds in oak leaves by high-performance ligand-exchange chromatography and electrospray ionization mass spectrometry for environmental stress studies. *J. Chromatogr., A* **2009**, *1216* (7), 1094–1099.

(48) Kim, J. K.; Bamba, T.; Harada, K.; Fukusaki, E.; Kobayashi, A. Time-course metabolic profiling in *Arabidopsis thaliana* cell cultures after salt stress treatment. *J. Exp. Bot.* **2007**, *58* (3), 415−424.