JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Nuclear Magnetic Resonance-Based Metabolic Comparative Analysis of Two Apple Varieties with Different Resistances to Apple Scab Attacks

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Supporting Information

ABSTRACT: Apple scab, caused by the fungus *Venturia inaequalis*, is the most serious disease of the apple worldwide. Two cultivars (*Malus domestica*), having different degrees of resistance against fungi attacks, were analyzed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Aqueous and organic extracts of both apple flesh and skin were studied, and over 30 metabolites, classified as organic acids, amino acids, carbohydrates, phenolic compounds, lipids, sterols, and other metabolites, were quantified by means of one-dimensional (1D) and two-dimensional (2D) NMR experiments. The metabolic profiles of the two apple cultivars were compared, and the differences were correlated with the different degrees of resistance to apple scab by means of univariate analysis. Levels of metabolites with known antifungal activity were observed not only to be higher in the Almagold cultivar but also to show different correlation patterns in comparison to Golden Delicious, implying a difference in the metabolic network involved in their biosynthesis.

KEYWORDS: *Malus domestica Borkh., NMR, metabolic profile, Venturia inaequalis*

INTRODUCTION

Apple (*Malus domestica* Borkh.) is one of the species in the Rosaceae family extensively cultivated and consumed worldwide. Over the centuries, apples have been selected to improve productivity, flavor, appearance, and storage life, but this has resulted in the loss of innate resistance of species to many diseases and pests.

Apple scab, caused by the ascomycetous fungus *Venturia inaequalis*, is the most serious disease of the apple worldwide.[1] It is so common in areas with wet spring weather that it is expected annually. *Venturia* spores, carried by wind on leaves and fruits of a plant, develop a germ tube able to penetrate the cuticle. The damages caused by apple scab are extensive, and in the worst cases, the entire crop could be lost. Moreover, severe defoliation could lead to a great productivity loss over a course of years as well as influencing its survival during winter conditions.

Apple is available in widely different cultivars; one of the most widespread and appreciated among them is the variety Golden Delicious, despite its high vulnerability to fungi attacks. Apple scab in commercial apple orchards is dealt through multiple pesticide treatments; more than 15 treatments with fungicide per season are generally required to prevent infection of a tree by *V. inaequalis* because they are to be repeated if late rains occur. This represents an important production cost and constitutes a questionable aspect in terms of environmental pollution and consumer health.[2]

For 2 decades, a new approach to reduce the impact of apple scab was developed, which is based on the breeding of scab-resistant cultivars. It was observed that some varieties showed a greater resistance toward *V. inaequalis* than others, and thus, a cross-breeding program between commercial and resistant cultivars was brought forth. More than 25 of these crosses have been released worldwide,[3−5] and in Italy, a farming program was started since 1994 within the research project of the Italian Ministry for Agricultural Policy "Fruit Varietal Orientation Lists"[6−8] to obtain scab-resistant apples. The Almagold[9] cultivar was obtained in 2007 through cross-breeding the varieties Ed Gould Golden and CO-OP 17. The latter had shown a resistance to fungi attacks but did not appear suitable to commercial distribution as a result of its poor sensorial properties. Almagold, instead, possesses a good balance between the resistance toward apple scab and good flavor and, as such, is a good candidate for widespread production.

The main objectives of the present study were to characterize the metabolic profile of Almagold apple, to compare this profile to the profile of the more vulnerable cultivar Golden Delicious, and to assess the presence of metabolites with potential

Received: July 7, 2015
Revised: September 3, 2015
Accepted: September 8, 2015
Published: September 8, 2015

DOI: 10.1021/acs.jafc.5b03311
J. Agric. Food Chem. 2015, 63, 8339−8347

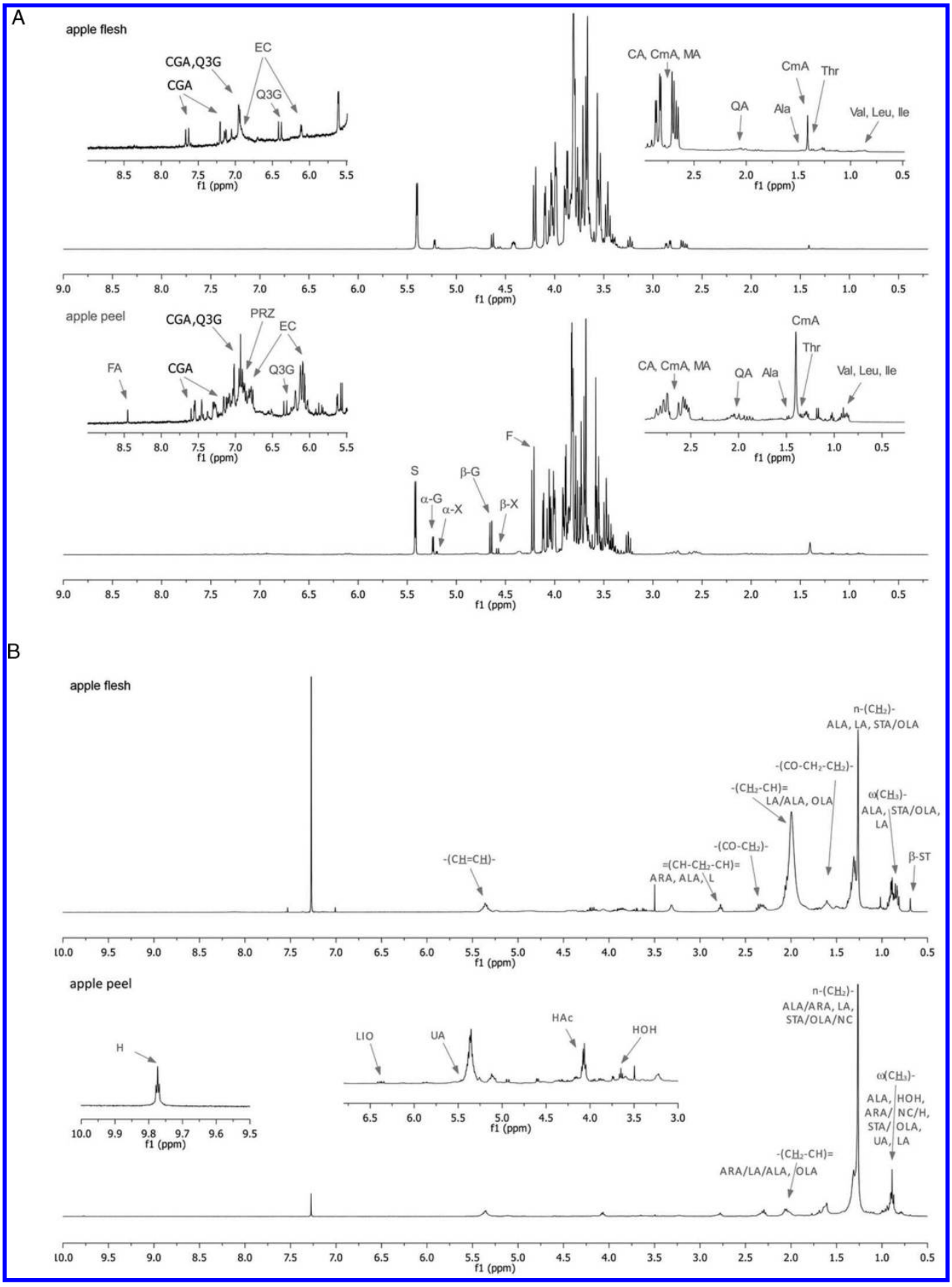

Figure 1. ^{1}H NMR spectrum of Almagold apple flesh (upper) and skin (lower) (A) hydroalcoholic and (B) chloroformic extracts at 298 K. The assignment of some resonances is reported.

DOI: 10.1021/acs.jafc.5b03311
J. Agric. Food Chem. 2015, 63, 8339−8347

antifungal activity by nuclear magnetic resonance (NMR) spectroscopy.

At present, an increasing importance is given to this technique[10] because it is able to point out different classes of chemical compounds, both endogenous and exogenous, at the same time, in a single spectrum, and it was successfully applied to several complex food matrices.[11−16] In particular, the comparison by NMR spectroscopy of cultivars of the same species with different degrees of resistance toward external attacks has already proven to be a reliable tool to detect molecules involved in fruit defense as well as their precursors.[12]

Because metabolites of flesh are closely related to fruit quality while those of peel are important in plant defense against fungal attacks, with the skin being a barrier between the fruit and the environment, hydroalcoholic and chloroformic extracts of both flesh and peel of the two apple cultivars were characterized by NMR spectroscopy.

MATERIALS AND METHODS

Sampling. Eight fruits of the Almagold cultivar (scab-resistant apple) and eight of the Golden Delicious cultivar (scab-susceptible apple) were hand-harvested in an organic farm orchard, located in Scurelle (Trento) in the northern Italian region Trentino-Alto Adige/Südtirol. The climate is temperate with significant rainfall during the year (annual average of 850 mm). The trees were 9 years old, grafted on M26 roostock, planted at a distance of 2.5 × 4.5 m, trained as "spindle", and irrigated with a drip irrigation system. The soil is classified sandy loam, sub-acid with a normal content of macro- and microelements. The harvest of fruits was made in the third decade of September at the stage of commercial maturity for both cultivars, which was assessed according to the following parameters: firmness of 6.4−6.9 kg/cm^2, soluble solid of 12.4−12.8 °Brix, and starch index of 2.5−3.0. These measurements were carried out following the official guidance on objective tests to determinate the quality of fruits.[17] The fruits, having a diameter of 70−75 mm, were taken from a single plant, in parts of the canopy well exposed to light to ensure higher and uniform quality characteristics. The yield has been regulated by manual thinning, leaving 70−80 fruits per tree. All apples were sampled in arbitrary order within a total time of 7 days, which were stored at 277 K before use. The selected fruits were without any apparent injury.

Sample Preparation. Slices of fresh cut flesh (about 1.2 g of fresh weight) were frozen in liquid nitrogen, finely powdered, and extracted according to the modified Bligh−Dyer methodology,[18] with a 2:2:1 methanol/chloroform/water final volumetric ratio. The sample was kept at 277 K for 1 h and then centrifuged for 20 min at 10000*g* at the same temperature. The upper hydroalcoholic phase and the lower organic phase were carefully separated and dried. The dried phases were stored at 193 K until the NMR analysis.

The peel was carefully separated from the flesh with the aid of a scalpel, pooled to acquire about 1.5 g of fresh material, and then underwent to the same extraction and drying procedures as the flesh.

Dry Matter Measurement. Dry matter was determined[17] by drying the apple sample in a oven at 70 °C until consecutive weightings made at 2 h intervals vary by less than 3 mg.

NMR Experiments. Each dry extract of the hydroalcoholic phase was dissolved in 0.6 mL of D_2O containing 2 mM 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt as the chemical shift and concentration reference.

Each chloroformic phase sample was resuspended in 0.6 mL of $CDCl_3$ containing 2 mM cyclohexamethyltrisiloxane as the chemical shift and concentration reference.

All solvents and standards were purchased from Sigma-Aldrich (St. Louis, MO).

All spectra were recorded at 298 K on a Bruker AVANCE III spectrometer operating at the proton frequency of 400.13 MHz and equipped with a Bruker multinuclear *z*-gradient inverse probehead.

Aqueous extract ^{1}H spectra were acquired employing the standard presat pulse sequence for solvent suppression with 64 transients, a spectral width of 6000 Hz, and 64 000 data points for an acquisition time of 5.5 s. The recycle delay was set to achieve a 15 s total acquisition time to avoid relaxation effects.

^{1}H spectra of the chloroformic phase samples were acquired with 64 transients with a spectral width of 6000 Hz and 32 000 data points for an acquisition time of 2.62 s. The recycle delay was set to achieve a 13 s total acquisition time to avoid relaxation effects.

Total correlation spectroscopy (TOCSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) two-dimensional spectra were acquired on a selection of samples to univocally assign ^{1}H and ^{13}C resonances. TOCSY experiments were acquired with a spectral width of 6000 Hz in both dimensions, a data matrix of 4000 × 256 points, a mixing time of 110 ms, and a relaxation delay of 2 s.

HSQC experiments were performed with a spectral width of 6000 and 25 500 Hz for the proton and carbon, respectively, a data matrix of 4000 × 256 points, an average $^1J_{C-H}$ of 145 Hz, and a recycle delay of 2 s.

Several HMBC spectra were acquired with a spectral width of 6000 and 25 500 Hz for the proton and carbon, respectively, a data matrix of 4000 × 256 points, long-range constants $^nJ_{C-H}$ of 4, 8, and 12 Hz, and a recycle delay of 2 s.

Quantification of the metabolites was performed by comparison of the signal integral to the reference integral, and quantities were expressed in milligrams per gram of fresh weight.

Statistical Analysis. The signals that could be clearly identified and had no overlap with neighboring signals were integrated for each sample. The resulting data set was submitted to analysis of variance (ANOVA) for the evaluation of statistical differences between the cultivars to point out the molecules responsible for the separation.

Data are presented as the mean ± standard deviation (SD). Univariate ANOVA was performed with Sigmaplot 11.0 software (Systat Software, Inc., San Jose, CA). A Shapiro−Wilk test was performed on each variable to assess their normality prior one-way ANOVA analysis. On the variables positive to ANOVA, a Holm−Sidak test, an all pairwise multiple comparison test, was applied to determinate which categories were discriminated by these metabolites ($p < 0.05$). Pearson correlation (Pearson product moment correlation) was carried out using Sigma stat software to observe the correlation between the quantified metabolites. The threshold value for correlation significance was $r = 0.765$. This value was chosen according to the sample population ($n = 8$) and α level = 0.05. Therefore, in this work, only the correlations with $r < -0.765$ and $r > 0.765$ were considered.

RESULTS AND DISCUSSION

Metabolic Profile. The comprehensive metabolic profile analysis of the two apple cultivars was carried out by ^{1}H NMR spectroscopy of hydroalcoholic and chloroformic extracts of both skin and flesh. Panels A and B of Figure 1 show representative ^{1}H NMR spectra (of aqueous and organic flesh and skin apple extracts) used for this purpose and the major NMR peaks that have contributed to the discrimination between the two cultivars. A total of 26 and 34 metabolites in flesh and skin, respectively, were identified. Their ^{1}H chemical shifts, multiplicity, and ^{13}C chemical shifts are reported in the Supporting Information.

Several NMR signals were identified by ^{1}H and ^{13}C NMR characteristic and cross-correlated signals in two-dimensional (2D) spectra as well as a comparison to literature data.[19,20] The assignment of the ^{1}H spectrum will be discussed for a class of compounds.

Hydroalcoholic Phase. Organic Acids. In the ^{1}H spectrum of aqueous flesh and skin apple extracts, acetic acid (AA), citric acid (CA), malic acid (MA), citramalic acid (CmA), succinic acid (SA), and quinic acid (QA) were identified by means of their diagnostic peaks and, with the exception of AA and SA,

DOI: 10.1021/acs.jafc.5b03311
J. Agric. Food Chem. 2015, 63, 8339−8347

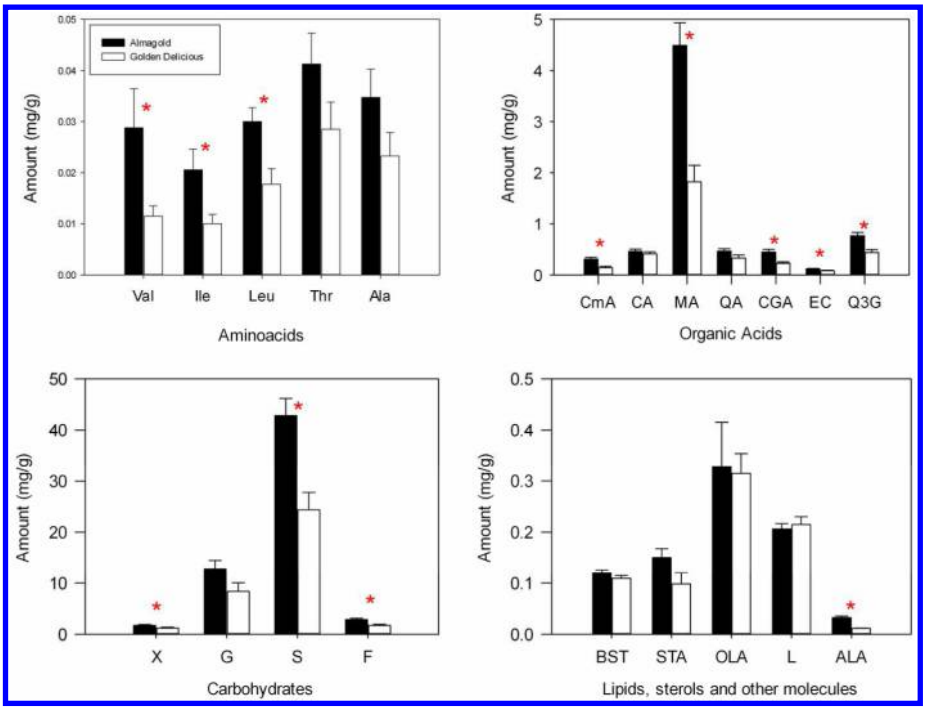

Figure 2. Amount of metabolites quantified by ^{1}H NMR in the flesh of Almagold (black bars) and Golden Delicious (white bars) apples. The metabolites positive to ANOVA ($p < 0.05$) are evidenced by an asterisk.

their TOCSY correlation patterns. CmA was univocally identified on the basis of its diagnostic singlet at 1.41 ppm (CH_3) and its doublets at 2.42 and 2.72 ppm (CH_2). The QA TOCSY spectrum showed scalar correlation among the signals at 1.89 and 2.09 ppm (CH_2-1), 2.00 and 2.06 ppm (CH_2-5), 3.55 (CH-3), 4.02 ppm (CH-2), and 4.15 ppm (CH-4).

Carbohydrates. The main carbohydrates detected in the ^{1}H spectrum of aqueous flesh and skin apple extracts were α- and β-glucose (G), α- and β-xylose (X), fructose (F), and sucrose (S). These molecules were identified on the basis of their diagnostic proton CH-1 anomeric doublets: α- and β-G at 5.25 and 4.69 ppm, respectively, α- and β-X at 5.18 and 4.57 ppm, S at 5.42 ppm, and CH-3 of F at 4.22 ppm. The remaining protons of the aforementioned spin systems were confirmed by means of 2D TOCSY experiments.

Free Amino Acids. The free amino acids leucine (Leu), isoleucine (Ile), valine (Val), threonine (Thr), and alanine (Ala) were identified by 2D TOCSY experiments of aqueous flesh and skin apple extracts. Their characteristic resonances are a multiplet at 0.97 ppm for Leu (δ,δ'-CH_3), a doublet at 1.02 ppm for Ile (γ-CH_3), a doublet at 1.05 ppm for Val (γ'-CH_3), a doublet at 1.33 ppm for Thr (γ-CH_3), and another doublet at 1.49 ppm for Ala (β-CH_3).

Phenolic Compounds. In the ^{1}H NMR spectrum of aqueous apple extracts, epicatechin (EC), chlorogenic acid (CGA), quercetin-3-*O*-galactoside (Q3G), and phloridzin (PRZ) were identified on the basis of their diagnostic spin systems. EC was identified through the TOCSY correlations between the resonances at 6.12 ppm (CH-6) and 6.14 ppm (CH-8), among the signals at 6.68 ppm (CH-6′), 6.75 ppm (CH-5′), and 6.95 ppm (CH-2′), and among the signals at 2.49 and 2.82 ppm (CH_2-4″), 3.97 ppm (CH-3″), and 4.56 ppm (CH-2″).

The caffeoyl moiety of CGA was univocally assigned as a result of the diagnostic doublets at 6.41 ppm (=CH−CO_2−) and 7.62 ppm (−CH=), while the QA moiety was assigned thanks to the TOCSY correlation pattern among the resonances at 2.07 and 2.22 ppm (CH_2-1′), 2.09 and 2.24 ppm (CH_2-5′), 3.88 ppm (CH-3′), 4.24 ppm (CH-4′), and 5.32 ppm (CH-2′).

Q3G aglycone was identified by TOCSY cross-peaks between the aromatic resonances at 6.20 ppm (CH-6) and 6.41 ppm (CH-8) and among the resonances at 7.69 ppm (CH-5′), 7.55 ppm (CH-6′), and 6.91 ppm (CH-2′) and its glycone was identified by the diagnostic doublet at 5.48 ppm (CH-1″).

PRZ was identified by TOCSY correlations between the resonances at 6.88 ppm (CH-3,5) and 7.15 ppm (CH-2,6) and the singlets at 6.05 ppm (CH-5′) and 6.21 ppm (CH-3′).

Organic Phase. Lipids. In the ^{1}H NMR spectrum of flesh and skin chloroformic phase, several lipid species were identified on the basis of TOCSY correlation patterns among the broad resonances at about 0.9 ppm (CH_3), 1.2 ppm (*n*-CH_2), 1.6 ppm (CO−CH_2−$C\underline{H}_2$), 2.0 ppm ($C\underline{H}_2$-CH=), 2.3 ppm (CO−$C\underline{H}_2$), 2.8 ppm (=CH−$C\underline{H}_2$−CH=), and 5.2 ppm (CH=CH). In comparison of the signals rising from CH_2 in α to the carboxyl group (2.3 ppm) to the signal from CH_2 in α to the unsaturations (2.0 ppm), it was possible to evaluate both the saturated and total unsaturated fatty acid contents. The amount of polyunsaturated fatty acids (PUFAs) was determined on the basis of linoleic (L) and linolenic (ALA) lipid diagnostic resonances at 2.77 and 2.82 ppm, respectively.

On the basis of the integrals of the aliphatic chain CH_2 and their ratio with the other fatty acid chain resonances, it was possible to assess that the average length of the chains is 18 carbon atoms. Therefore, the fatty acids were quantified in equivalents of stearic acid (STA), oleic acid (OLA), linoleic acid (L), and linolenic acid (ALA).

Arachidonic acid (ARA) was detected and quantified as a result of its diagnostic resonance at 2.92 ppm.

Sterols. β-Sitosterol (β-ST) was univocally assigned on the basis of the HSQCs between hydrogen at 0.68 ppm (CH_3-18) and carbon at 12.2 ppm and between the resonances at 1.01

DOI: 10.1021/acs.jafc.5b03311
J. Agric. Food Chem. 2015, 63, 8339−8347

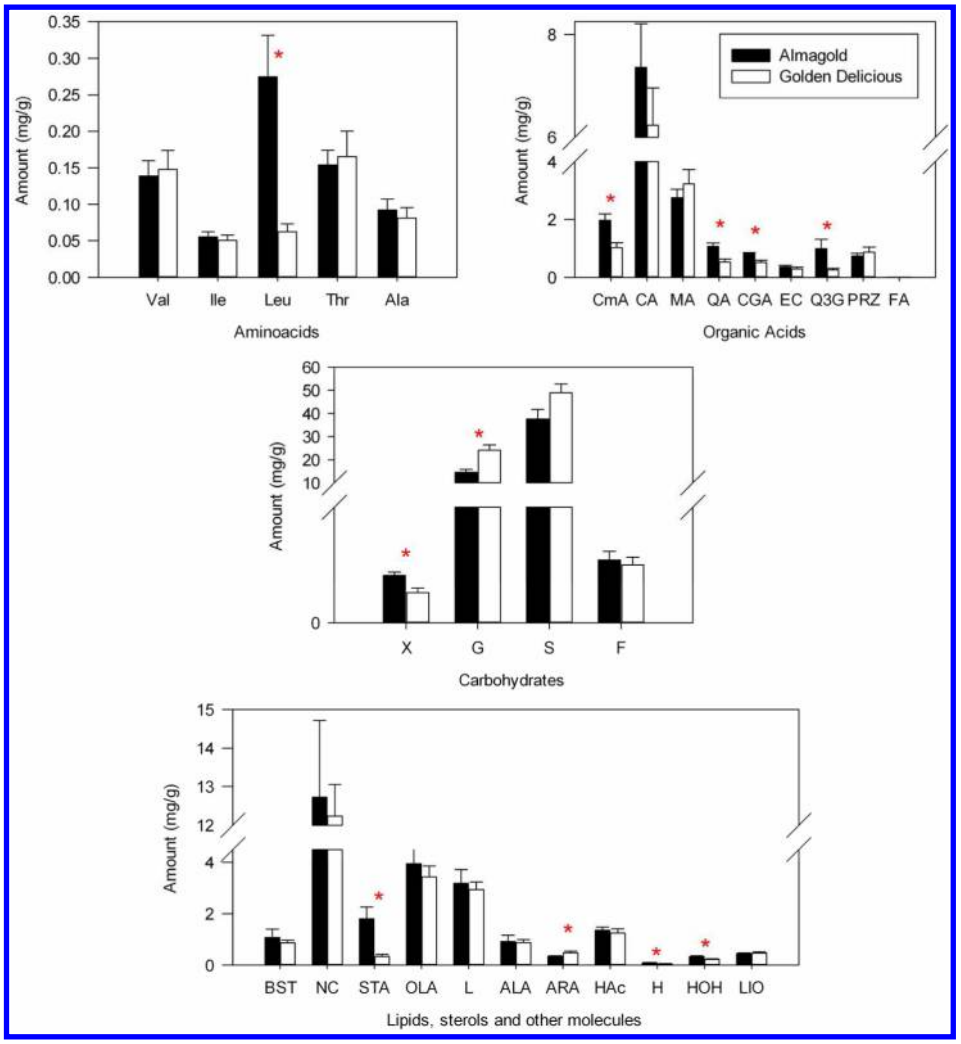

Figure 3. Amount of metabolites quantified by ^{1}H NMR in the skin of Almagold (black bars) and Golden Delicious (white bars) apples. The metabolites positive to ANOVA ($p < 0.05$) are evidenced by an asterisk.

ppm (CH_3-25) and 19.1 ppm. Other molecule resonances were identified on the basis of the TOCSY correlation pattern among the resonances at 5.34 ppm (CH-6), 1.98 and 1.52 ppm (CH_2-7), 1.46 ppm (CH-8), 0.99 ppm (CH-14), 1.57 ppm (CH_2-15), and 1.85 and 1.26 ppm (CH_2-16). Another TOCSY pattern that was attributed to the A ring of this sterol was formed by the proton signals at 3.52 ppm (CH−OH-3), 2.28 ppm (CH_2-4), 1.84 and 1.51 ppm (CH_2-2), and 1.85 and 1.08 ppm (CH_2-1).

Ursolic acid (UA) was univocally identified by HSQC and HMBC experiments. Some of the most diagnostic correlations were detected between the singlet at 5.49 ppm and the carbon signal at 125.7 ppm (CH-12), between hydrogen at 1.97 ppm and carbon at 37.4 ppm (CH_2-22), and between the protons at 1.61 ppm and carbon at 31.1 ppm (CH_2-21). Moreover, long-range correlations were observed between the protons 21 and 15 and carbon at 179.7 ppm (C-28).

Miscellaneous. Along with the aforementioned lipid species, several long-chain aliphatic molecules, such as nonadecane (NC), were observed in the ^{1}H NMR spectrum of the apple skin chloroformic phase. Their resonances were observed at 0.89 ppm (ω-CH_3) and 1.27 ppm (n-CH_2) and were distinguished from the similar lipid signals by their HSQCs as well as the absence of TOCSY correlations with the proton resonances of CH_2 in α and β positions with respect to the carboxyl group. These molecules are one of the main constituents of the waxy cuticle coating the apple skin and, along with other analogue linear olefins, are responsible for the relative adhesion of water, pesticides, fungal spores, and other airborne deposits on the fruit surface.

Linalool (LIO), hexanol (HOH), hexyl acetate (HAc), and hexanal (H) were detected only in the skin. LIO was identified on the basis of its diagnostic vinyl moiety resonance at 6.33 ppm (dd, =CH-2) and TOCSY correlation pattern with the resonances at 5.10 and 5.35 ppm (CH_2= 1a and 1b, respectively). Their complete assignment was performed by HSQC and HMBC experiments. Long-range HMBCs were observed between the H-2 and carbon in position 3 at 72.1 ppm, between this carbon and hydrogen at 0.96 and 1.21 ppm (CH_3-9 and CH_2-4, respectively), and between the quaternary carbon at 130.9 ppm (C-7) and the protons at 1.61, 1.68, and 5.08 ppm (CH_3-8, CH_3-10, and =CH-6, respectively). The resonance of proton in position 5 was univocally assigned on the basis of HSQC between its signal at 2.08 ppm and the carbon signal at 39.5 ppm and by HMBC between the aforementioned proton resonance and the carbon at 124 ppm (C-6).

DOI: 10.1021/acs.jafc.5b03311
J. Agric. Food Chem. 2015, 63, 8339−8347

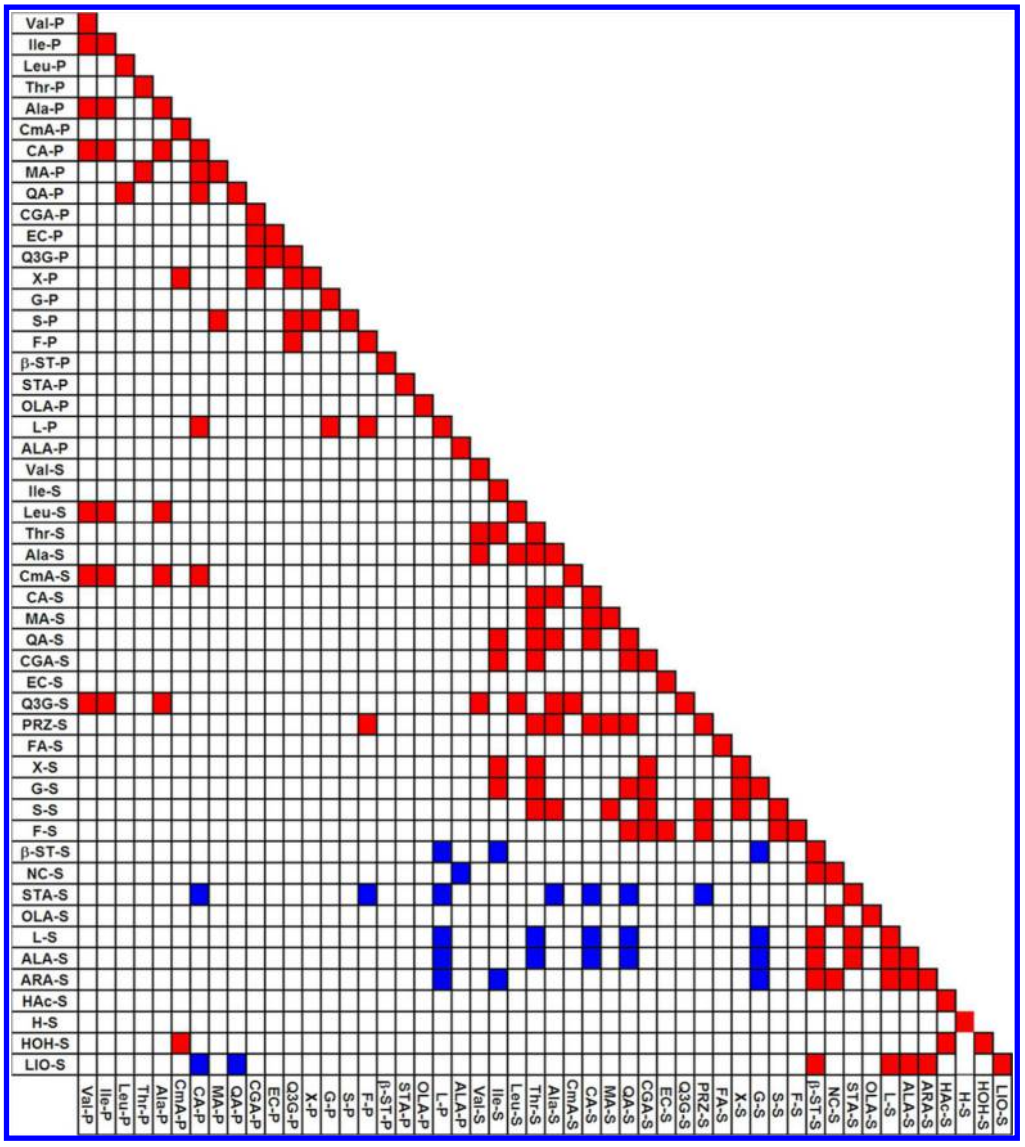

Figure 4. Pearson's correlation map of Almagold metabolites. Positive correlations are in red, while negative correlations are in blue. The correlation threshold is ±0.765.

The quantitative analysis is reported in Figures 2 and 3 for the flesh and peel metabolites, respectively.

Dry Matter Measurement. The measured dry matter content for Almagold samples was 16.7 ± 0.2%, while for Golden Delicious samples, it was 14.8 ± 0.3%. These data are in good agreement with NMR measurements for the apple flesh (Figure 2), because the metabolites present in Almagold are generally more abundant than those in the other cultivar.

Statistical Analysis. With regard to flesh analysis, the Almagold showed statistically ($p \leq 0.05$) larger amounts of Val, Ile, Leu, CmA, MA, CGA, Q3G, X, S, F, and ALA. With regard to peel analysis, the Almagold showed statistically ($p \leq 0.05$) larger amounts of Leu, CmA, QA, CGA, Q3G, X, and STA, while Golden Delicious contained larger amounts of G and ARA (Figures 2 and 3).

Because ANOVA is an univariate statistical analysis tool, this technique is unable to evidence correlations between the variables. These correlations are an important source of information because the ratios between the metabolites in a vegetal or animal sample are influenced by the metabolic network that is characteristic of each species or cultivar.

To quantify these correlations, Pearson coefficients were calculated and reported in Figures 4 and 5 for Almagold and Golden Delicious, respectively.

Comparison between Almagold and Golden Delicious. The metabolic profile comparison between the two apple varieties showed only quantitative differences (Figures 2 and 3) between metabolites, while both qualitative and quantitative differences were clearly highlighted between flesh and skin.

Most fruits and plants react to external attacks and pathogens producing several secondary metabolites to repel infections only after the first contact.[21] Nevertheless, it was observed that some fruits are able to resist against fungal attacks during quiescence not only on the basis of the synthesis of these defense metabolites after the infection but also because of the presence of preformed antifungal compounds.[22] Moreover, another benefit of pre-existent antimycotic metabolites is that, even in the case of a successful infection, those molecules were observed to reduce the secretion of fungal pathogenicity factors occurring during quiescent infections.[23]

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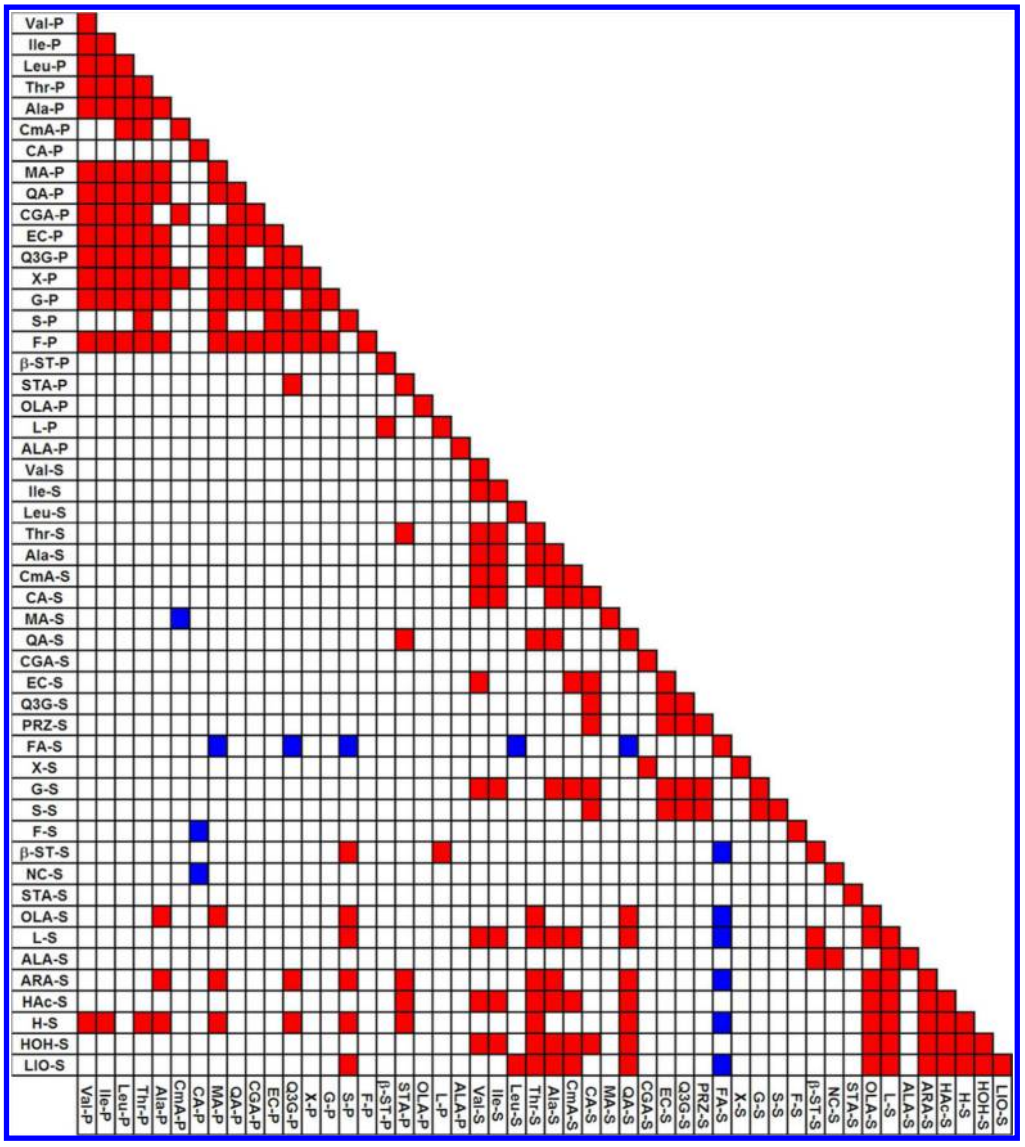

Figure 5. Pearson's correlation map of Golden Delicious metabolites. Positive correlations are in red, while negative correlations are in blue. The correlation threshold is ±0.765.

Skin Hydroalcoholic Phase. As stated before, statistical analysis performed on the skin metabolites shows that the Almagold cultivar, which is resistant to *V. inaequalis*, has a higher amount of Leu, CmA, QA, CGA, Q3G, and X and a lower amount of G (Figure 3), while metabolites such as EC and PRZ are not discriminant between the two cultivars. Among these molecules, QA, Q3G, and CGA play an important role in fruit defense against external threats, such as fungi attacks. In particular, QA is an intermediate of the phenylpropanoid pathway,[24] and it could be transformed into several different secondary metabolites according to external stimuli.

Q3G, the most abundant aromatic compound in both cultivars, is a flavonoid with a potent and non-specific antifungal activity found in many fruits and leaves,[24] was shown to be able to reduce the growth of several fungal species up to 99%, yet was never tested on *Venturia* strains specifically.

CGA is a powerful antioxidant, and high concentrations of this molecule in immature fruits were reported to aid the plant in repelling brown rot infection by interfering with fungal melanin production.[25] The same defense mechanism could be effective against other types of fungal attacks.

PRZ, detected only in the skin, is a glycosylated flavonoid that is involved in defense against herbivores and fungi attacks.[26] Nevertheless, this metabolite is present in almost the same amount in both cultivars (Figure 3), suggesting that this molecule is not directly involved in the protection against *V. inaequalis*.

In comparison of the skin metabolites of the two cultivars, it is also possible to observe that the total carbohydrate amount of Almagold is statistically lower than the total carbohydrate amount present in the Golden Delicious skin and that the sensible variety is especially rich in glucose. Fungi are unable to photosynthesize carbohydrates as a result of the lack of chloroplasts, and they need to absorb nutrients from other plants. Because the cultivar more vulnerable to *V. inaequalis* attacks, the Golden Delicious, is the cultivar with more carbohydrates in the skin, it is possible that the presence of readily available nutrients for the fungi could increase the infection diffusion in the fruit.

Skin Organic Phase. Several molecules, in particular, UA, LIO, NC, ARA, HOH, HAc, and H, were observed to be present only in the fruit skin.

DOI: 10.1021/acs.jafc.5b03311
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Quantitative differences were detected between the two cultivars, in particular, the Almagold is richer in H, HOH, and STA, while the Golden Delicious has greater amounts of ARA.

UA is a pentacyclic triterpene whose biological role is the protection against microbial and fungi attacks.[27] LIO is a monoterpene found in many flowers and greatly contributes to the pleasant scent.[28] NC, other analogue linear olefins, and to a lesser extent, ARA are some of the main constituents of the waxy cuticle coating the apple skin, and they are responsible for the adhesion of water, pesticides, fungal spores, and other airborne deposits on the fruit surface. Moreover, PUFAs are also known to act as antimycotic agents because they fluidize the fungi cell membrane[29] and sensitize them against other antifungal agents. Because ARA is more abundant in the more sensible cultivar, it is possible that this defense mechanism is not viable against the *V. inaequalis* strain or that its antifungal efficiency is lower that the efficiency observed for L and ALA fatty acids.

Among the observed *n*-hexane derivatives, H possesses antifungal activity,[30] and its presence in the fruit skin can lead to a faster response toward detected fungi attacks.

Flesh Hydroalcoholic Phase. With regard to the flesh hydroalcoholic phase, the more resistant cultivar possesses a higher amount of CmA, MA, CGA, and Q3G, but only the last two molecules are known to be involved in the protection against fungal attacks. Their presence in the flesh of Almagold could be important because some defense compounds are produced in flesh,[31] with the purpose of a "last ditch defense" against damages, such as the damages produced by a successful *V. inaequalis* infection.

With regard to other molecules with known antifungal activity, it is interesting to observe that the amount of QA is statistically higher in the skin than in the flesh of both cultivars, but while, in the skin, it was more abundant in Almagold apples, in the flesh, there were no statistical differences between the two species.

Flesh Organic Phase. In comparison of the composition of flesh organic phases, the main difference between the two cultivars is that Almagold has a higher amount of ALA fatty acids. In addition to their aforementioned direct antifungal activity, it is worth noting that PUFAs are the precursors for the biosynthesis of volatile linear chain molecules and the main type of volatiles in apples are C_6 linear and branched alcohols, aldehydes, and acids.[32]

Pearson Correlations. The comparison of the Pearson's correlation maps of the two cultivars highlights several interesting differences regarding the metabolic correlations of the molecules with defensive roles. First of all, correlations of the same metabolite between the two locations (i.e., Ala in the flesh and Ala in the skin) were not observed in either cultivar. The absence of a direct correlation between the concentration of a metabolite in the flesh with the same in the skin suggests that no exchange occurs between the two molecule pools. This hypothesis implies that the metabolites involved in the defense from external attacks, which are those found in the skin, are synthesized *in loco*.

Moreover, it can also be observed that, in the peel of Golden Delicious (Figure 5) variety, PRZ, CGA, Q3G, and EC correlate only with molecules located in the skin and not with the molecules of the flesh, whereas in the Almagold (Figure 4) variety, PRZ, Q3G, and EC correlate with molecules located in both flesh and peel. These metabolites are involved in several defense mechanisms and, as reported before, are synthesized both prior and after an external stimulus.[21,22] Other molecules, in the peel, that show different correlations between the two cultivars are QA and CGA. This is important because variations in Pearson's correlation patterns could suggest that the metabolic pathways involved in their synthesis and their following reactions are different between the two cultivars.

This study, given its general approach, shows how the NMR-based characterization of the metabolic profile is a promising tool for the rational choice of cultivars for selective cross-breeding aimed to the development of disease-resisting fruit varieties. In particular, it is possible to attribute the resistance toward *V. inaequalis* to higher contents of QA, CGA, and Q3G and a minor amount of free carbohydrates in the peel of the fruit. Nevertheless, it is important to highlight that another major difference between the resistant and vulnerable cultivars lies in the correlation patterns among the levels of the molecules responsible for defense; this indicates that their biosynthetic pathways are different between the two cultivars, and this difference could also contribute to the different degrees of reaction to fungi attacks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b03311.

Table of the metabolites identified in the ^{1}H NMR spectrum of the aqueous and chloroformic extracts of apples (PDF)

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Funding

The present work has been carried out under the project "Research for the Improvements of the Fruit Trees Protected Cultivation in Southern Italy" funded by the Italian Ministry of Agriculture and Forestry Policy.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AA, acetic acid; Ala, alanine; ALA, linolenic acid; ARA, arachidonic acid; β-ST, β-sitosterol; CA, citric acid; CGA, chlorogenic acid; CmA, citramalic acid; EC, epicatechin; F, fructose; FA, formic acid; G, glucose; H, hexanal; HAc, hexyl acetate; HOH, hexanol; Ile, isoleucine; L, linoleic acid; Leu, leucine; LIO, linalool; MA, malic acid; NC, nonadecane; OLA, oleic acid; PRZ, phloridzin; PUFA, polyunsaturated fatty acid; QA, quinic acid; Q3G, quercetin-3-*O*-galactoside; SA, succinic acid; S, sucrose; STA, stearic acid; Thr, threonine; UA, ursolic acid; Val, valine; X, xylose

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