¹H NMR-Based Metabolomics Reveals a Pedoclimatic Metabolic Imprinting in Ready-to-Drink Carrot Juices

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Ⓢ Supporting Information

ABSTRACT: Carrots are usually consumed in their native form or processed into many different products. Carrot juice is a popular beverage consumed throughout the world and is attracting increasing attention due to its nutritional value, being a natural source of bioactive compounds. Ready-to-drink carrot juices produced in the same factory were analyzed by ¹H nuclear magnetic resonance (NMR) spectroscopy. The juices were made from carrot roots of the same cultivar grown in three different geographical areas in Italy. More than 30 compounds have been identified and quantified, and the data was subjected to univariate ANOVA and multivariate analyses. Clear geographical-dependent clustering was observed, and the metabolic profiles were related to the different pedoclimatic conditions. The proposed phytoprofiling approach could be employed on an industrial scale to evaluate finished products involving different sites of supply of the raw material, thus improving both the quality and uniformity of the juices.

KEYWORDS: NMR spectroscopy, metabolic profiling, Daucus carota L., carrot juice
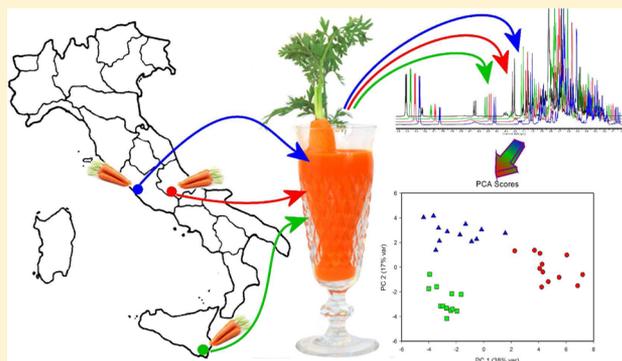
■ INTRODUCTION

The growing attention consumers are paying to healthier diets has contributed to increased interest in the global food sector of "natural and healthy" products, such that manufacturers are now aiming at improving their products in terms of quality, safety, and nutritional properties. The beneficial health effects claimed for regular consumption of unprocessed vegetables or as a finished product represent a challenge for the food industry to provide products with guaranteed nutritional and sensory qualities.

Carrots (*Daucus carota* L.) are one of the most important and cheapest vegetable crops in the world[1] and are recognized worldwide as a source of different compounds with documented anti-inflammatory, antioxidant, antimicrobial, antiviral, and anticancer properties.[2−4] Carrot consumption has increased in recent years, either in their native form or processed into ready-made products such as powdered soups, tinned and frozen foods, concentrates, and, in particular, in the form of juices either alone or mixed with other vegetables and fruits. The excellent quality of taste, sweetness, and flavor of the juice together with its nutritional properties such as high carotenoid, vitamin, and mineral content and low energy values explains its high consumer appreciation.[4−7] However, as part of a general problem with plant-derived foods, the chemical composition of the basic carrot raw material is affected by factors such as different cultivars, geographical origin, growing

sites, seasonal and climatic differences, and storage of the root before processing.[8,9] Furthermore, it is widely known that industrial processing of juice production may significantly alter the compositional properties due to the possible loss of several valuable nutrients.[10,11]

Some investigations have followed a metabolomic approach focused mainly on the characterization of botanical species and cultivars in the form of raw materials, semifinished products, or finished products, defining a new discipline known as "Foodomics" that studies foods and nutrition via the application of omic technologies.[12−15] In particular, multivariate analysis methods applied to ¹H NMR spectra have been used to identify the geographical origin of products like wine, tea, beer, olive oil, or tomato paste, among others[16−23] and even microgeographical variations were recently demonstrated in green teas obtained from leaves cultivated at different altitudes.[24]

In this regard, a metabolomic approach has proven its value in assessing genotypic and phenotypic diversity in plants, compositional comparisons, defining biochemical changes associated with plant growth, as well as evaluating the quality,

processing, and safety of both the starting raw material and the final products obtained.[12−15]

In particular, NMR spectroscopy has been applied to the study of sugars and anthocyanine compounds in carrots,[25] and variations in the metabolomes of genetically different carrots were revealed using NMR-based metabolomics.[26]

In the present study, the metabolome of carrot juices was explored with a view to describing the general differences in chemical composition of final products processed using raw material from different geographical sites of cultivation. This is because the short growing time obliges producers to supply the raw material from different areas to ensure steady availability throughout the year, thus introducing further variability of the starting material. To minimize the variability of the samples, juices were analyzed utilizing carrots of the same cultivar as raw material, grown and processed identically in the same factory with the sole variability of being ascribed to three different growing sites in Italy.

By using ^{1}H NMR spectroscopy and multivariate statistical analysis, it was possible to differentiate the juices obtained from the different cultivation areas according to their metabolite content. Furthermore, it was possible to relate the PCA structured groups to the pedoclimatic conditions of the three different carrot growing sites.

■ MATERIALS AND METHODS

Chemicals. Deuterium oxide, deuterated chloroform, (trimethyl-silyl)-propionic-2,2,3,3,-d_4 acid sodium salt (TSP-d_4), cyclohexame-thyltrisyloxane (CHMTSO), chloroform, and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sampling. Carrot juice was provided by Aureli Mario S.S. Agricola (Ortucchio, AQ, Italy) in sterile bags as a finished product. The same carrot cultivar (*Daucus carota* L., Nantese Dordogne, Syngenta seeds) was sown and harvested at commercial maturity as indicated by the supplier's geneticists; furthermore, random samples of carrot roots were evaluated in terms of other maturation criteria (length, uniformity of color, crispness, root apical morphology) over the maturation period. The carrots were grown in fields at three different Italian sites: Maccarese (Lazio), Ispica (Sicily), and Fucino (Abruzzo). Maccarese (sea level; 41°52′47″N, 13°38′47″E) has a warm and temperate climate with an average temperature of 3−15 °C in winter and 16−30 °C in summer with a relative humidity of 30%. Ispica (sea level; 36°46′06″N, 14°54′05″E) has a climate similar to that of Maccarese, warm and temperate with an average temperature of 9−15 °C in winter and 19−30 °C in summer but with a much higher relative humidity (75%). Fucino (altitude 750 m; 41°52′47″N; 12°12′36″E) has a considerably colder climate with an average temperature of −3 to 9 °C in winter and 9−29 °C in summer with a relative humidity of 61%.

The raw material was refrigerated (4 °C) during delivery and storage and was processed at day three from harvesting to ensure uniformity. The carrot roots were washed, mill ground, extracted, pasteurized, and aseptically packaged. The pasteurized carrot juices were delivered to the laboratory in sterile bags at 4 °C. Twelve bags were collected for analysis from the production pipeline every half hour on two consecutive processing days for each geographical origin. The production pipeline processes some 10,000 kg/hour of raw material; therefore, each juice collected reflected a very large sampling of carrots.

Sample Preparation. Twelve aliquots for each type of carrot juice were extracted following a modified Bligh−Dyer protocol.[27] Briefly, 4.5 mL of a cold mixture composed of chloroform and methanol in a 1:2 ratio and 1.5 mL of cold chloroform (final 2:2:1 chloroform:me-thanol:juice) were added to 1.5 mL of carrot juice. The samples were stirred, stored at 4 °C overnight, and then centrifuged for 20 min at 10,000*g* at 4 °C. The upper hydrosoluble phases and the lower organic phases were carefully recovered and dried by fluxing with N_2. The

dried phases were stored at −80 °C for NMR analysis. The hydrosoluble phase was then resuspended in 600 μL of a mixture of D_2O and CD_3OD (2:1 ratio) containing 3-(trimethylsilyl)-propionic-2,2,3,3,-d_4 acid sodium salt (TSP, 2 mM) as internal chemical shift and concentration standard. The organic phase was resuspended in 600 μL of $CDCl_3$ containing cyclohexamethyltrisyloxane (CHMTSO, 2 mM) as a concentration reference. All solvents and standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

NMR Experiments. Monodimensional ^{1}H NMR spectra of the hydroalcoholic phases were performed by collecting 64 scans for each spectrum on a Bruker Avance 400 spectrometer (Bruker Spectrospin, Karlsruhe, Germany) operating at 9.4 T at 298 K using a 90° detection pulse and acquiring the FIDs in 64 K points; the spectral width was 6009.15 Hz. The solvent signal was suppressed by means of a presaturation scheme, and the relaxation delay was set to 6.55 s to achieve a 15 s total acquisition time so as to avoid relaxation effects.

Monodimensional ^{1}H NMR spectra of the chloroform phases were acquired by collecting 64 scans using a 90° detection pulse and acquiring the FIDs in 32 K points over a spectral width of 6009.15 Hz and a recycle delay of 10.28 s to achieve a total acquisition time of 13 s to avoid relaxation effects.

Homonuclear [1]H−[1]H TOCSY and heteronuclear [1]H−^{13}C HSQC and HMBC bidimensional experiments were performed on a few samples of both phases to ensure signal assignment. TOCSY experiments were acquired with a spectral width of 6009.15 Hz in both dimensions, a data matrix of 8 K × 256 points, a mixing time of 110 ms, and relaxation delay of 2 s. HSQC experiments were performed with a spectral width of 6009.15 and 25500 Hz for the proton and carbon, respectively, with a data matrix of 8 K × 256 points, an average $^1J_{C−H}$ of 145 Hz, and a recycle delay of 3 s. Several HMBC spectra were acquired with a spectral width of 6009.15 and 25500 Hz for the proton and carbon, respectively, with a data matrix of 8 K × 256 points, long-range constants $^nJ_{C−H}$ of 4, 8, and 12 Hz, and a recycle delay of 3 s.

Monodimensional ^{1}H spectra were analyzed with 1D NMR Manager software ver. 12 (ACD/Laboratories, Toronto, Canada): FIDs underwent exponential multiplication (LB = 0.09 Hz), were Fourier transformed, and were phase and baseline corrected. Baseline correction was automatically performed through the built-in software "FID Reconstruction" function that eliminates the broad baseline components in the time domain before the Fourier transform. Bidimensional spectra were processed with Bruker Topspin ver. 2.1.

The assignment of the resonances was performed by analyzing ^{1}H and ^{13}C NMR characteristics and cross-correlated signals in 2D spectra (TOCSY, HSQC, and HMBC spectra) by comparison with literature compilations[28,29] and, when necessary, by adding standard com-pounds. Quantification of the metabolites was performed by comparison of the signal integral with the reference one, and quantities were expressed in millimolar concentration (mM) of metabolites in the juice.

Multivariate Analysis. Multivariate data analyses were performed with Unscrambler ver. 9.8 software (Camo Software AS, Oslo, Norway). Data were mean centered and autoscaled to avoid the effect due to the wide differences among the metabolite concentrations and to equalize the importance of the variation of each variable. Principal component analysis (PCA) was applied.[30]

Univariate Analysis. Univariate analysis of variance (ANOVA) was performed with Sigmaplot 12.0 software (Systat Software Inc., San Josè, California). The assessment of data normality was performed by the Shapiro-Wilk test. Holm-Sidak, an all pairwise multiple comparison test, was applied to determine which categories were discriminated by these metabolites ($p < 0.05$).

■ RESULTS

Metabolic Profiling. The comprehensive metabolic profile analysis of carrot juices from different geographic regions was carried out by ^{1}H NMR spectroscopy of hydroalcoholic and chloroformic extracts. Figures S1A and S1B show representative ^{1}H NMR spectra of hydroalcoholic and organic extracts,

respectively. The juices from the three different cultivation sites showed quantitative but no qualitative differences. A total of 37 metabolites were identified and quantified. ^{1}H chemical shifts, multiplicity, and ^{13}C chemical shifts have been summarized in Table S1. The assignments will be discussed by class of compounds.

1. Compound Identification: Hydroalcoholic Phase. 1.1. Organic Acids. In the ^{1}H spectrum of hydroalcoholic carrot extracts, isovaleric acid (IVA), lactic acid (LA), acetic acid (AA), citric acid (CA), malic acid (MA), fumaric acid (FumA), quinic acid (QA), and formic acid (FA) were identified by means of their diagnostic peaks and, with the exception of AA, FumA, and FA, their TOCSY correlation patterns. IVA was unequivocally identified on the basis of its diagnostic doublet at 0.90 ppm (CH$_3$) and its multiplets at 1.95 (CH) and 2.05 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 (CH-2), and 4.15 ppm (CH-4) (Figure S3). The complete assignment of QA structure using HSQC and HMBC (Figures S4 and S5, respectively) was reported as an example of the methodology adopted.

1.2. Carbohydrates. The carbohydrates detected were α and β glucose (G), fructose (F), and sucrose (S). These molecules were identified on the basis of their diagnostic CH-1 anomeric proton doublets α- and β-G at 5.25 and 4.68 ppm, respectively, at 5.42 ppm for S, and at 4.21 ppm for CH-3 of F. The remaining protons of the aforementioned spin systems were confirmed by means of 2D TOCSY experiments.

1.3. Free Amino Acids. The free amino acids leucine (Leu), isoleucine (Ile), valine (Val), threonine (Thr), alanine (Ala), glutamine (Gln), aspartate (Asp), asparagine (Asn), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), and γ-aminobutyric acid (GABA) were identified by 2D TOCSY experiments. 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$_3$), a doublet at 1.49 ppm for Ala (β-CH$_3$), a triplet of doublets at 2.45 ppm for Gln (β-CH$_2$), a doublet of doublets at 2.72 and 2.89 ppm for Asp and Asn, respectively (β'-CH), a multiplet at 7.22 ppm for Tyr (CH-2,6), a multiplet at 7.42 ppm for Phe (CH-3,5), a multiplet at 7.75 ppm for Trp (CH-4), and a multiplet at 1.95 ppm for GABA (β-CH$_2$).

1.4. Miscellaneous Compounds. In the ^{1}H NMR spectrum of hydroalcoholic extracts, choline (Chn), catechin (Cat), quercetin-3-*O*-glucoside (Q3G), uridine phosphate (UXP), and niacinamide (Nam) were identified on the basis of their diagnostic spin systems. Chn was identified by its diagnostic singlet at 3.20 ppm (N(CH$_3$)$_3$) and confirmed by HSQC experiments. Cat was identified through the TOCSY correlations between the resonances at 6.02 (CH-6) and 6.04 ppm (CH-8), among the signals at 6.68 (CH-6'), 6.75 (CH-5'), and 6.95 ppm (CH-2') and among those at 2.49, 2.82 (CH$_2$-4''), 3.97 (CH-3''), and 4.56 (CH-2'') ppm. Q3G aglycone was identified via TOCSY cross peaks between the aromatic resonances at 6.20 (CH-6) and 6.41 ppm (CH-8) and among those at 7.69 (CH-5'), 7.55 (CH-6'), and 6.91 ppm (CH-2') and its glycone by the diagnostic doublet at 5.48 ppm (CH-1''). UXP was identified on the basis of its doublet at 5.97 ppm (CH-5) coupled with its other doublet at 7.89 ppm (CH-6). Because it is not possible to identify the resonances of the different uridine phosphate compounds, the UXP concen-

tration has been expressed as equivalents of uridine monophosphate molecules.

Nam was recognized on the basis of the broad singlet at 8.91 ppm (CH-2), and its other resonances at 8.78 (CH-6), 8.21 (CH-4), and 7.69 ppm (CH-5) were identified by TOCSY correlations.

2. Compound Identification: Organic Phase. 2.1. Lipids. In the ^{1}H NMR spectrum of the chloroformic phase, several lipid species were identified on the basis of TOCSY correlation patterns among the broad resonances at approximately 0.9 (CH$_3$), 1.2 (*n*-CH$_2$), 1.6 (CO−CH$_2$−CH$_2$), 2.0 (CH$_2$−CH$=$), 2.3 (CO−CH$_2$), 2.8 ($=$CH−CH$_2$−CH$=$), and 5.2 (CH$=$CH) ppm. Comparing the signals arising from CH$_2$ in α to the carboxyl group (2.3 ppm) with that from the CH$_2$ in α to unsaturation (2.0 ppm), it was possible to evaluate both the saturated and total unsaturated fatty acid content. The amount of polyunsaturated fatty acids was determined on the basis of linoleic (L) and linolenic (ALA) lipid diagnostic resonances at 2.76 and 2.82 ppm, respectively. From the integrals of the aliphatic chain CH$_2$ and their ratio to the other fatty acid chain resonances, it was possible to determine that the average length of the chains is 18 carbon atoms. Therefore, the fatty acids were quantified as equivalents of stearic acid (SFA), oleic acid (OLA), linoleic acid (L), and linolenic acid (ALA).

β-Sitosterol (β-ST) was univocally assigned on the basis of the HSQC correlations between the hydrogen at 0.68 ppm (CH$_3$-18) and the carbon at 12.20 ppm and between the resonances at 1.01 (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 (CH-6), 1.98 and 1.52 (CH$_2$-7), 1.46 (CH-8), 0.99 (CH-14), 1.57 (CH$_2$−15), and 1.85 and 1.26 ppm (CH$_2$-16). Another TOCSY pattern, which was attributed to the A ring of this sterol, was formed by the proton signals at 3.52 (CH−OH-3), 2.28 (CH$_2$-4), 1.84 and 1.51 (CH$_2$-2), and 1.85 and 1.08 ppm (CH$_2$-1). The other identified sterol, campsterol (Cmp), was identified via the HSQC correlation between the singlet at 0.70 ppm (CH$_3$-18) and the carbon at 12.21 ppm. Its other protons were attributed on the basis of their TOCSY correlation patterns.

2.2. Other Molecules. Falcarinol (Flc) was identified on the basis of its diagnostic spin system at 5.95 (CH-2), 5.47 (CH-1a), 5.26 (CH-1b), and 4.92 ppm (CH-3), whereas its other spin system was detected through TOCSY correlation among the resonances at 5.52 (CH-10), 5.38 (CH-9), 3.05 (CH$_2$-8), 2.03 (CH$_2$-11), 1.35 (CH$_2$-12), 1.28 (CH$_2$-13 through -16), and 0.89 ppm (CH$_3$-17). Because both falcarinol and falcarindiol resonate at the same chemical shift, Flc concentration was calculated as equivalents of falcarinol.

Carotenoid (Crt) resonances were assigned on the basis of their several TOCSY correlation patterns among the resonances at 6.66 (CH-11,11'), 6.35 (CH-12,12'), and 6.14 ppm (CH-10,10'); between the multiplets at 6.63 (CH-15,15') and 6.25 ppm (CH-14,14'); between the signals at 6.15 (CH-7,7') and 6.14 ppm (CH-8,8'); and among those at 2.02 (CH$_2$-4,4'), 1.62 (CH$_2$−3,3'), and 1.47 ppm (CH$_2$−2,2'). Their singlets at 1.97 (CH$_3$-19,19'), 1.72 (CH$_3$-18,18'), and 1.03 ppm (CH$_3$-16,16',17,17') were identified through their HSQC correlations with the carbons at 12.8, 21.77, and 29.01 ppm, respectively. Because several different types of carotenoids resonate at the same chemical shifts, Crt concentration was calculated as equivalents of β-carotene.

Table 1. Concentrations of the Metabolites in Raw Carrot Juice[a]

	concentration (mM)			
	Ispica	Maccarese	Fucino	
		Amino Acids		
valine	0.38 ± 0.02	0.35 ± 0.01	0.65 ± 0.09	A**, B**
isoleucine	0.24 ± 0.01	0.25 ± 0.01	0.39 ± 0.05	A**, B**
leucine	0.17 ± 0.01	0.18 ± 0.02	0.25 ± 0.03	A*, B*
threonine	0.34 ± 0.03	0.26 ± 0.02	0.49 ± 0.08	B**, C*
alanine	1.90 ± 0.12	1.23 ± 0.03	3.46 ± 0.59	A**, B**,C**
GABA	1.45 ± 0.07	1.99 ± 0.07	1.91 ± 0.19	A*, C**
glutamine	2.20 ± 0.10	2.78 ± 0.10	2.21 ± 0.24	B*, C**
aspartate	0.72 ± 0.03	0.88 ± 0.04	1.37 ± 0.18	A**, B**, C**
asparagine	0.94 ± 0.03	1.42 ± 0.08	2.66 ± 0.36	A**, B**, C**
phenylalanine	0.11 ± 0.01	0.06 ± 0.00	0.14 ± 0.03	B**, C**
tyrosine	0.12 ± 0.01	0.16 ± 0.01	0.37 ± 0.06	A**, B**,C**
tryptophan	0.11 ± 0.01	0.11 ± 0.01	0.20 ± 0.03	A*, B**
		Organic Acids		
isovalerate	0.11 ± 0.01	0.12 ± 0.01	0.17 ± 0.02	A*, B*
lactate	0.06 ± 0.01	0.20 ± 0.02	0.51 ± 0.09	A**, B**, C**
acetate	0.14 ± 0.01	0.23 ± 0.01	0.85 ± 0.20	A**, B**, C**
quinate	1.30 ± 0.07	1.59 ± 0.09	3.21 ± 0.52	A**, B**, C*
citrate	1.51 ± 0.13	1.64 ± 0.18	4.39 ± 0.62	A**, B**
fumarate	0.26 ± 0.02	0.38 ± 0.03	0.24 ± 0.02	B**, C**
formate	0.09 ± 0.01	0.05 ± 0.00	0.06 ± 0.01	A*, C**
malate	12.87 ± 0.48	19.60 ± 1.80	19.10 ± 1.50	A**, C**
		Carbohydrates		
glucose	43.00 ± 2.00	68.20 ± 2.00	46.60 ± 4.20	B**, C**
sucrose	85.20 ± 5.00	85.80 ± 2.20	86.30 ± 8.70	
fructose	5.35 ± 0.48	4.63 ± 0.34	3.17 ± 0.34	A**, B**
		Sterols and Fatty Acids		
β-sitosterol	0.28 ± 0.00	0.30 ± 0.01	0.31 ± 0.02	C**
campsterol	0.07 ± 0.00	0.09 ± 0.00	0.08 ± 0.00	B*, C**
stearic acid	1.92 ± 0.07	2.42 ± 0.06	2.11 ± 0.17	C**
oleic acid	0.15 ± 0.02	0.22 ± 0.02	0.30 ± 0.05	A**, C**
linoleic acid	1.50 ± 0.04	2.17 ± 0.05	2.16 ± 0.15	A**, C**
linolenic acid	0.24 ± 0.01	0.31 ± 0.01	0.27 ± 0.02	C**
		Miscellaneous		
choline	0.27 ± 0.01	0.52 ± 0.01	0.46 ± 0.04	A**, C**
quercetin glycoside	0.29 ± 0.01	0.70 ± 0.04	0.46 ± 0.04	A**, B**, C**
catechin	0.10 ± 0.01	0.13 ± 0.01	0.25 ± 0.05	A**, B**
uridine phosphate	0.20 ± 0.01	0.24 ± 0.01	0.29 ± 0.03	A**, C*
niacinamide	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	A**, C**
falcarinol	0.44 ± 0.02	0.52 ± 0.02	0.58 ± 0.04	A**, C**
carotenoids	0.24 ± 0.01	0.26 ± 0.01	0.33 ± 0.01	A**, B**

[a] A indicates statistically different levels between Ispica and Fucino juices, B between Maccarese and Fucino, and C between Ispica and Maccarese. *P < 0.05 and **P < 0.01.

Quantification of the metabolites was performed by signal integration. Because of the overcrowding of ^{1}H NMR spectra, only those signals that did not overlap with other resonances were integrated. Quantities were expressed as millimolar concentration of metabolite (mM) in carrot juice. The concentration was determined by comparing the diagnostic resonance integral of each molecule (Table S1) with the reference signal both normalized for the number of protons generating the resonance (9 protons for TSP and 18 protons for CHMTSO).

The quantitative analysis is reported in Table 1 with the statistical significance determined by ANOVA.

For evaluating the presence of latent variables correlating the metabolite concentrations, PCA analysis was performed on the metabolite quantities. PCA analysis provided a model in which the first two principal components accounted for 55% of the overall variance (38 and 17%, respectively). Their score plot (Figure 1) displays three groups, each corresponding to a single geographic origin. PC1 separates Fucino carrots from the other two origins, and PC2 differentiates between Maccarese (higher levels) and Ispica carrots. The loading plot (Figure 1) shows that PC1 is characterized by high values of citrate, aspartate, tyrosine, quinic acid, and isoleucine along with low values of fructose. PC2, instead, shows high levels of camptosterol and quercetin and low levels of phenylalanine.

Loading plot analysis allowed juices from all geographic origins to be differentiated. The differences are mainly due to the different amounts of amino acids and organic acids. The juices obtained from carrots harvested at Fucino showed statistically significantly increased levels of all the detected free
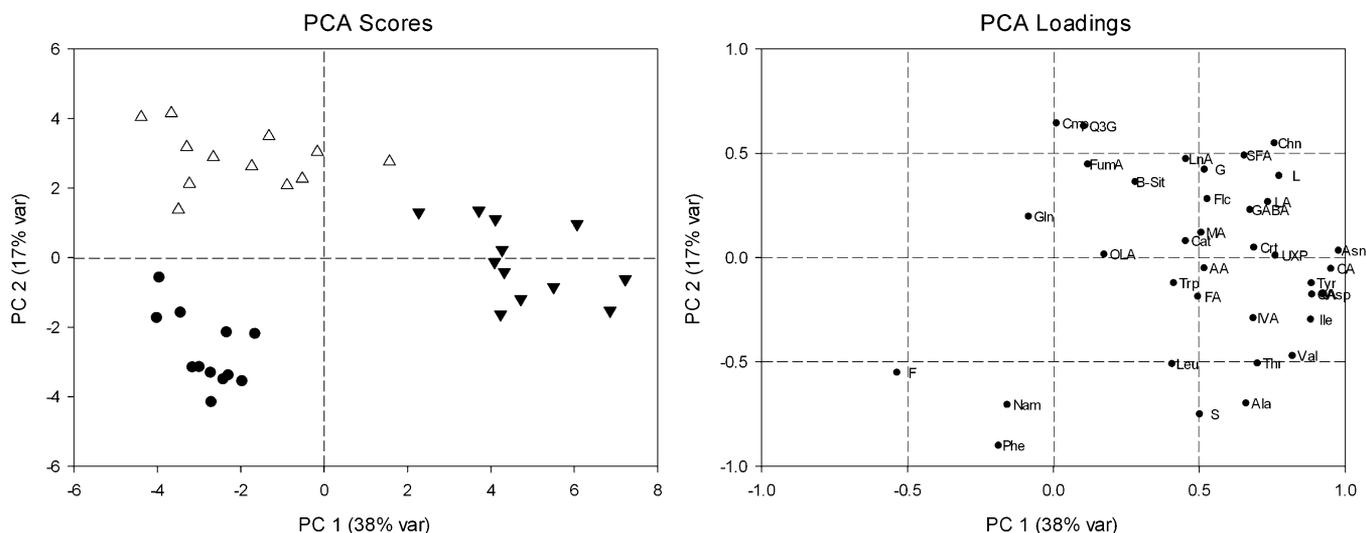
Figure 1. PCA score plot (left) and PCA loading plot (right) of carrot juices are reported (●, Ispica; Δ, Maccarese; ▼, Fucino). The first two principal components explained 55% of the total variance.

amino acids with the exception of glutamine (Table 1). Interestingly, the measured levels were nearly twice as high in Fucino juices as their levels in either Ispica or Maccarese juices or both. Additionally, amino acid levels differed significantly between Ispica and Maccarese, although only for glutamine, asparagine, and GABA, which displayed lower levels, and alanine and phenylalanine, which displayed higher levels in Ispica versus Maccarese juices.

A quite similar trend was detected for isovalerate, lactate, acetate, quinic acid, and citrate, which were 2- to 5-times higher in Fucino juices. Formate levels were the highest in Ispica juices where the lowest levels of lactate, acetate, and fumarate were recorded. Sucrose and fructose represented by far the most and least abundant sugars in the three juices and highest and lowest levels in Fucino juices, respectively. The sucrose levels were not different between Maccarese and Ispica juices, whereas glucose and fructose levels were the highest in Maccarese and Ispica juices, respectively.

Quercetin content differed significantly in the three juices, showing the highest levels in Maccarese juices and the lowest in Ispica juices. The highest levels of choline, uridin phosphate, and catechin were measured in Fucino juice with the latter compound having a level nearly 3-times higher than in both Maccarese and Ispica juices. No statistical differences in the levels of β-sitosterol and camptosterol were observed in the juices from the three cultivation sites, whereas the levels of stearic, oleic, linoleic acid, and linolenic acid were the lowest in Ispica juices. Falcarinol and carotenoids displayed an increasing concentration trend in Ispica, Maccarese, and Fucino juices with significant differences measured for carotenoids in Fucino juices with respect to the other two and for falcarinol only for Ispica versus Fucino juices.

■ DISCUSSION

The quality control of a finished product can be fundamental for consumer loyalty toward a specific brand taking into account that a food processing industry is forced to acquire raw material from different cultivation sites to ensure uninterrupted production. On the other hand, great variability has been widely demonstrated in the levels of nutrients in plants depending on cultivar, developmental stage, harvest time, and pedoclimatic

conditions. In this regard, a metabolomic approach represents a powerful method for providing a comprehensive profile of the biochemical composition starting from the raw material and up to the ready-to-consume product, passing through the processing step.[31−34]

In the present study, the influence of three different cultivation sites on the phytonutrient profiles of ready to drink carrot juices was investigated using ^{1}H NMR-based metabolic profiling. For the intrinsic variability of the raw material to be minimized, the same carrot cultivar, cultivation methods, and harvesting time were used. Furthermore, the processing into juices was performed at the same factory as differential changes in the final composition are known to be influenced by this technical step.[4,35,36] Here, it was shown that the proposed method has the potential to highlight the influence of latitude, elevation, and pedoclimatic conditions in terms of biochemical components even in ready-to-drink juices. Indeed, the growing sites are matched in pairs for altitude (Maccarese and Ispica at sea level with respect to Fucino at 750 m) and latitude (Fucino and Maccarese at 41°N with respect to Ispica at 36°N) while the longitude difference is within 3°E degrees.

The uniformity of the raw material in terms of genetics (same cultivar) and processing was reflected in the core of the primary metabolites, such as sugars, amino acids, and organic acids, which were found to be unchanged among the juices obtained from carrots grown in the different areas. Conversely, the levels of several components displayed substantial variation up to 2- to 5-times higher for amino acids and organic acids in Fucino-derived juices.

PCA analysis applied to the NMR data showed a clear clustering of the three types of juices as a function of the geographical origin of the raw material. In particular, carrot juices from the Fucino highland were strongly characterized by high levels of some amino acids and citric acid and a low level of fructose with respect to those of Ispica and Maccarese juices. The increased levels of the free amino acids contained in Fucino juices suggested the involvement of a temperature acclimation process in a colder climate. An accumulation of branched-chain amino acids as precursors of secondary metabolites has been observed in response to both heat and

cold stress in *Arabidopsis* and in abiotic stresses in general as part of a defense response against pathogens in a weakened host.[37−39]

In agreement with our results, the growing temperature emerged as a critical factor for both sensory and chemical variables, and variation in the chemical composition of carrots as a function of the north-to-south direction of the growing site was observed in four carrot varieties in Norway.[40]

The role of a colder climate in carrot composition was also suggested by the increased level of GABA, a four carbon non-protein amino acid in the Fucino-derived juices. Indeed, an increase in GABA intra- and extracellular concentrations has been observed in response to a range of biotic and abiotic stresses through the regulation of pH, carbon fluxes in the TCA cycle, and osmoregulation. Interestingly, glutamate decarboxylase 1 (GAD1), an enzyme of the GABA shunt, is root specific, and 90% of the genes downregulated by GABA are preferentially expressed in the roots.[38,41,42]

Interestingly, our metabolomics analysis revealed citrate as one of the main metabolites involved in the geographic-dependent clustering of the carrot juices. Higher levels of malate, which represents the organic acid having the highest concentration in carrots, and of citrate in Fucino-derived juices were recorded, and an increase in the levels of other organic acids, namely isovalerate, lactate, and acetate, depending on the altitude of the growing site was observed. In agreement with this, an upregulation of the citric acid cycle to maintain homeostasis at chilling temperatures has been demonstrated.[37] It is known that both the types and the levels of organic acids vary according to plant species, tissue type, and developmental stage.[43−45] Because organoleptic characteristics, palatability, and sourness have been linked to acidity, the possibility of obtaining a comprehensive measure of all of the organic acids could represent an added value of the proposed approach for manufacturers in the choice of growing site and harvest time of the relevant crops.

Although the composition of the soil was not investigated in the three different growing sites, our results could also suggest a soil-related effect as several studies have shown downregulation of citrate catabolism in lupin cluster roots and an accumulation of citrate and malate in roots as a function of the mineral content of the rhizosphere.[46,47] The increase in carboxylates would precede their release in the root exudate whose qualitative and quantitative composition is influenced by different environmental factors, including soil temperature and nutrient availability.[47]

A soil-dependent effect is further suggested by the measured levels of flavonoids in the juices. It is an accepted fact that the flavonoid content and composition in roots is highly dependent on the geographical location, plant species, developmental stage, and biotic and abiotic environmental conditions, and their exudation has been shown to increase in response to abiotic stresses, including temperature and soil characteristics.[48,49] Furthermore, flavonoid and citrate release has been shown to be temporally regulated with regard to root maturation in lupins.[50] Although the sum of the measured flavonoids in the three juices did not show substantial variation, the levels of quercetin and catechin were significantly different with the latter compound displaying a level nearly three times higher in Fucino juices than in both Maccarese and Ispica juices, whereas quercetin was the highest and lowest in Maccarese and Ispica juices, respectively. Flavonoids are synthesized starting from phenylalanine to naringenin in plants

with the pathway diverging at this step forming the different classes of compounds.[49] In agreement with this, our data could suggest a soil- and/or temperature-dependent effect on both carboxylates and flavonoid contents of the raw material that can still be measured in the processed juices.

Finally, a pedoclimatic influence was suggested by the different levels of quinic acid, polyacetylenes, and carotenoids, all of which represent compounds with verified health-promoting effects. In particular, quinic acid, whose level was increased more than 2-fold in Fucino juices, is a precursor of phenolics, a class of compounds with high antioxidant properties widely distributed in plant foods, including carrots. Interestingly, a dependence of both polyacetylenes, namely falcarinol and falcarindiol, and carotenoid content in carrot roots on cultivar, growing conditions, storage, and processing has been demonstrated.[3,36,51,52]

To our knowledge, this is the first study in which a direct pedoclimatic influence on the bioactive compound concentration has been evidenced as the same cultivar, growing conditions, storage, and processing were used. Furthermore, the levels of polyacetylenes should be kept in consideration by the manufacturers because falcarindiol content has been suggested to contribute to the bitterness of carrot juices.[5] However, a sensory panel failed to detect a more bitter taste in Fucino juices in which the unpleasant taste could have been masked by the higher content of sucrose.

In conclusion, it was demonstrated that a ^{1}H NMR-based metabolomic approach could highlight the pedoclimatic related biochemical differences ("terroir" effect) directly in the ready-to-drink carrot juices. The proposed phytoprofiling approach could be very useful for manufacturers to evaluate their finished products relative to different sites of supply of the raw material, thus ensuring a uniform quality potentially leading to a "golden standard" of a single brand. Information concerning the processing techniques employed and the discovery of unintended differences in metabolite composition and/or contents could ensure a high level of internal quality control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01555.

> Table S1, resonance assignment list; Figure S1, ^{1}H NMR spectrum of the hydroalcoholic phase of carrot juice extracts; Figure S2, ^{1}H NMR spectrum of the chloroformic phase of carrot juice extracts; Figure S3, extract of the TOCSY [1]H−[1]H bidimensional experiment of carrot juice hydroalcoholic extract; Figure S4, extract of the HSQC [1]H−[3]C bidimensional experiment of carrot juice hydroalcoholic extract; Figure S5, extract of the HMBC [1]H−[3]C bidimensional experiment of carrot juice hydroalcoholic extract (PDF)

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