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Article

Amino acid content in onions as potential fingerprints of geographical origin: the case of *Rossa da Inverno sel. Rojo Duro*

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Abstract: In the frame of a broader project, we were interested at comparing the amino acid profile in a specific variety of onion, *Rossa da inverno sel. Rojo Duro*, produced in two different Italian sites: Cannara (Umbria region) and Imola (Emilia Romagna region). In both places, onions were cultivated and harvested in the same way, and irrigated by water sprinkler method. A further group of Cannara onions, growth by microirrigation, was also evaluated. After the extraction of free amino acid mixture from onion samples, an ion-pairing RP-HPLC method allowed the separation and the evaporative light scattering detection of almost all underivatized proteinogenic amino acids. However, only the peaks corresponding to Leu, Phe, Trp, were present in all the investigated samples and unaffected from matrix interfering peaks. The application of the beeswarm/box plots with the ANOVA/TukeyHSD statistical approach revealed a content of Leu and Phe markedly influenced by the geographical origin of the onions, while not by the irrigation procedure. The developed HPLC method was validated in terms of specificity, linearity, LOD and LOQ, accuracy and precision, before the quantitative assay of Leu, Phe and Trp in the onion samples. Although further studies are necessary, these preliminary findings can represent a good starting point for considering the quantity of specific amino acids in the *Rossa da inverno sel. Rojo Duro* variety as a fingerprint of its geographical origin. In principle, the developed approach might be applied to other onion varieties, thus contributing to their characterization and traceability, also contributing to limit commercial frauds.

Keywords: *Rossa da inverno sel. Rojo Duro* onion *cultivar*; geographical origin; amino acids content; HPLC analysis; statistical evaluations; food traceability

1. Introduction

Onions (*Allium cepa L.*) are the second most used vegetable worldwide after tomatoes [1]. A continuous interest is directed to the selection of the varieties and to the production of fresh and processed products with defined organoleptic and healthy properties. Onions are a valuable source of phenolic substances, especially quercetin and its glycosides, sulphur compounds, phenolic acids, vitamins and minerals, while a limited content of amino acids is present. Nevertheless, it is known that amino acids contribute to the sensory response and to the characteristic taste called 'umami' [2].

We have long been interested in the study and definition of the properties of onions from Cannara, a small town in Umbria region (Italy) [3-5]. In particular, in the frame of a broader project, we were interested, *inter alia*, at comparing the amino acid content in a specific variety of onion (*Rossa da inverno sel. Rojo Duro*) produced in two different locations, Cannara (group A) and Imola (Emilia Romagna region, Italy, group B). In both places, onions were cultivated and harvested in the same way, and irrigated by water sprinkler method.

The amino acid content was appraised by using an Ion Pair - Reversed Phase High Performance Liquid Chromatography (IP-RP HPLC) methodology with the aid of an Evaporative Light Scattering Detector (ELSD). A further group of Cannara onions (group C), growth using water microirrigation, was also taken into account in the setting of the study.

The role of amino acid analysis in food chemistry is well-recognized, not only to assess product biological value, but also as a characterization parameter of different food sources [6-9].

In general, as far botanical species are concerned, the evaluation of specific metabolites composition could be used as a criterion to evaluate the proceedings of production of a particular variety, pointing out a plausible relationship with the growing location, the soil and the weather conditions [10,11]. Accordingly, the appraisal of type and levels of these metabolites could provide useful information about the variability in terms of organoleptic and nutritional properties [12-14].

During the study we observed that the levels of some amino acids were different between the samples from Cannara and Imola. Accordingly, in the present work, we tried to assess a relationship between the content of these amino acids and the geographical origin of the onion cultivar, thus contributing to favor the food traceability.

2. Results and Discussion

The amino acid pool in the lyophilized samples was extracted with deionized water according to the procedure described in section 3.4. The amino acid profile was then determined chromatographically by means of a previously established IP-RP HPLC method [15].

It is worth to recall that heptafluorobutyric acid (HFBA) as IP reagent increases the analyte lipophilicity, its retention in RP settings, and hence the quality of the chromatographic performance in terms of selectivity and efficiency. Moreover, differently from other perfluoroalkyl carboxylic acids, HFBA containing eluents give the advantage to avoid prolonged re-equilibration times between consecutive runs [15]. This ultimately facilitates the rapid analysis of numerous samples.

Not less important, HFBA is volatile and compatible with mass spectrometry (MS) detectors for accurate molecular investigations.

With the use of a non-polar end-capped RP-18 column, and a 7 mM HFBA concentration in the aqueous eluent component (see section 3.5 for details), the optimized gradient program produced a noticeable chromatographic performance towards the separation of a standard pool of the most representative underivatized proteinogenic amino acids. Consequently, the established method was applied to the extracts. The exemplary chromatogram of a real sample is shown in Figure 1.

On the basis of the comparison between the retention times of the peaks in each analyzed extract, with those of a standard amino acid mixture, the following amino acids were easily identified and the accurate mass then confirmed through High Resolution Mass Spectrometry (HRMS) analysis (data not shown): threonine (Thr), alanine (Ala), glutamic acid (Glu), valine (Val), arginine (Arg), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), and tryptophan (Trp). Unfortunately, during the analysis of many extracts, the co-elution of some of the above amino acids with unidentified matrix deriving peaks occurred. Only the peaks corresponding to the three amino acids Leu, Phe, Trp, were found in all the investigated samples and fully resolved from other peaks in the chromatogram.

Therefore, these compounds were considered suitable for further analyses and quantifications.

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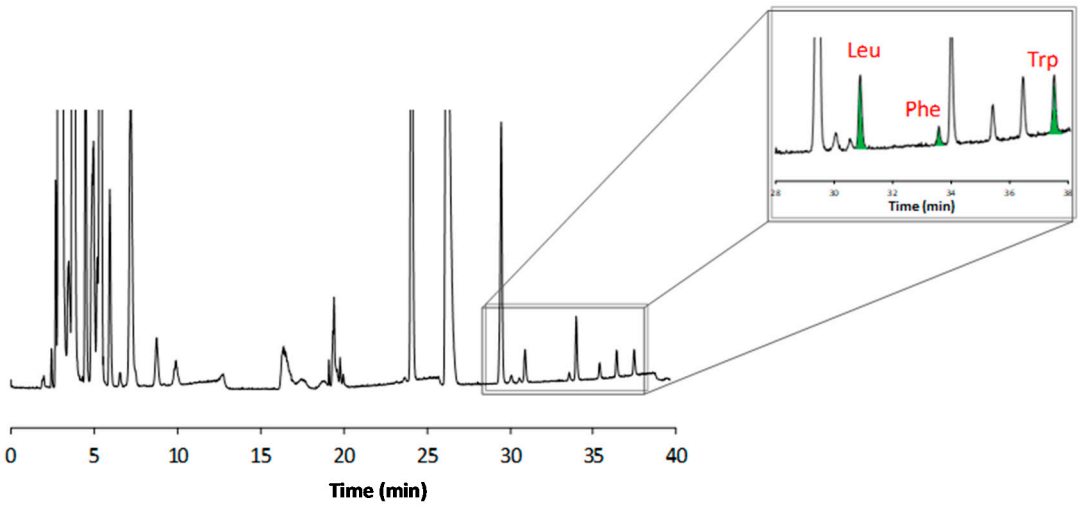

Figure 1. Chromatogram of an extracted sample. On the top right, the enlarged section of the chromatogram in the time-window containing the three amino acids Leu, Phe, Trp is highlighted. Y-axis is in mV scale.

2.1. Method Validation and Amino Acid Quantification

The content of the selected amino acids in the extract samples was determined by using the external calibration method, by correlating the logarithm peak area vs the logarithm analyte concentration values [16]. Usually, when an ELSD is used, a non-linear (almost always exponential) relationship between the output signal (area value, A) and the corresponding analyte concentrations (m) occurs (equation 1) when a wide range of concentrations is explored [17-19].

$$A = am^b \quad (1)$$

In all these cases, the logarithm transformation is the common way to linearize the exponential profile of area vs concentration values plots (Figure 2).

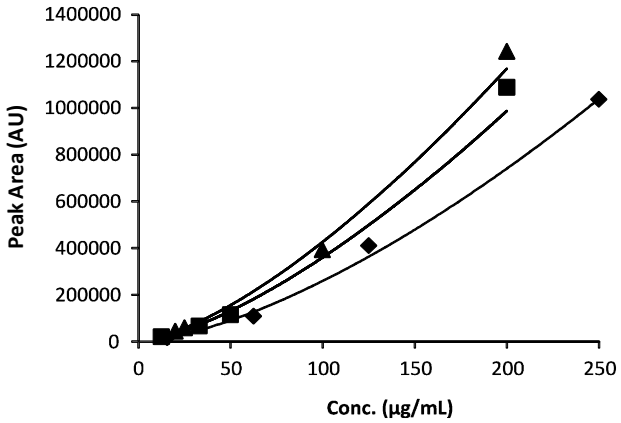

Figure 2. Calibration curves obtained for the three selected amino acids (◆ Leu; ■ Phe, ▲ Trp).

By employing the general equation (2), three calibration curves were thus obtained in the present study, with appreciably high R^2 values (Table 1).

$$\log A = b \log m + \log a \quad (2)$$

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The regression equations reported in Table 1 were used to validate the chromatographic method and for quantitative analyses. Appreciably low LOD and LOQ values were calculated for the investigated amino acids. The method was also validated for precision and accuracy, in both the short- (intra-day) and the long-term (inter-day) period.

Table 1. Calibration data for the selected amino acids: regression equations, correlation coefficient (R^2) values, explored linearity ranges, LOD and LOQ values.

AA	Regression eq.	R^2	Linearity conc. range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Leu	y= 1.52(± 0.07)x + 2.38(±0.14)	0.9951	15.6-250	0.15	0.44
Phe	y= 1.45(±0.08)x + 2.65(±0.144)	0.9940	12.5-200	1.44	2.95
Trp	y= 1.45(±0.04)x + 2.72(±0.07)	0.9984	25-200	0.08	0.23

As reported in Table 2, a very profitable precision of the method was diagnosed in the short period. Accordingly, a comparable and low range of variation of the RSD% values (from 0.53 up to 9.5%) was observed during the consecutive three days of analysis, thus ensuring a profitable stability of our analytical method. In accordance to this outcome, acceptable RSD% values (ranging from 4.71 to 10.51%) were also recorded when the long term (inter-day) precision was evaluated (Table 3).

The percentage of the recovery, the so-called "Recovery test" [20], was employed to estimate the accuracy of the IP-RP HPLC-ELSD method. As reported in Tables 2 and 3, acceptable percentages of recovery were obtained: in the case of the intra-day analyses ranging from 84.08 up to 118.20 (Table 2), whereas during long-term runs from 90.49 to 105.16 (Table 3).

Table 2. Statistical analysis for the three selected amino acids in the short period (intra-day precision and accuracy values).

AA	Solution	Day	Theoretical conc. (μg/mL)	Mean observed conc. (μg/mL)	n[a]	Precision (RSD%)	Accuracy (Recovery%)
Leu	1	1	31.20	27.89	3	1.05	89.38
		2		28.64		9.50	91.81
		3		31.09		3.82	99.66
	2	1	160.00	161.54	3	2.83	100.96
		2		150.92		5.80	94.32
		3		171.65		4.69	107.28
Phe	1	1	25.00	21.02	3	3.75	84.08

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		2		23.52		6.36	94.07
		3		23.55		2.12	94.22
		1		118.20		0.53	118.20
	2	2	100.00	100.79	3	9.12	100.79
		3		96.49		2.83	96.49
		1		28.43		3.35	86.15
	1	2	33.00	28.87	3	2.81	87.48
		3		32.29		4.15	97.84
Trp		1		135.64		4.90	94.85
	2	2	143.00	137.36	3	2.62	96.06
		3		145.90		3.41	102.03

[a] Number of replicates.

Table 3. Statistical analysis for the three selected amino acids in the long period (inter-day precision and accuracy values).

AA	Solution	Theoretical conc. (μg/mL)	Mean observed conc. (μg/mL)	n[a]	Precision (RSD%)	Accuracy (Recovery%)
	1	31.20	29.21		7.13	93.61
Leu				9		
	2	160.00	163.94		6.72	102.46
	1	25.00	22.70		6.77	90.79
Phe				9		
	2	100.00	105.16		10.51	105.16
	1	33.00	29.86		6.86	90.49
Trp				9		
	2	143.00	139.63		4.71	97.65

[a] Number of replicates.

The excellent results achieved in the validation step, prompted us to apply the HPLC method for the content determination of the selected amino acids in an extended set of onion samples (groups A-C, see section 3.3. and 3.4 for details).

Based on the regression equations in Table 1, the average concentrations of the three amino acids were calculated and the data shown in Table 4.

Table 4. Means ± SEM of concentration values determined for the selected amino acids of interest in the three groups studied (A-C). SEM is for "standard error of the mean".

Group	Mean ± SEM conc. (μg/mL)		
	Leu	**Phe**	**Trp**
A	37.4±13.4	10.6±2.7	20.0±7.4
B	49.9±13.5	16.3±5.0	31.4±6.6
C	41.1±11.3	11.9±3.6	22.2±4.6

As clearly evident from data in Table 4, the concentrations of three amino acids from the group B (samples from Imola) are greater than those found in the other two groups (A and C: onion samples collected in Cannara).

2.2. Statistical Evaluation

In order to highlight differences in the content of Leu, Phe, Trp in samples with different geographical origin (groups A and B), a further and depper statistical evaluation was performed.

Many known plots are available and used to show distributions of univariate data. Tukey introduced the box and whiskers plot as part of his toolkit for exploratory data analysis [21]. These are particularly useful for comparing distributions across groups when other statistical methods such as ANOVA and Tukey HSD are employed. Furthermore, to visualize the data point on the box plot representation, a beeswarm plot was also implemented. Indeed, the superimposition of both plots is useful to gain a very rich description of the underlying distribution.

By following this statistical approach, the obtained data relatively to the Leu, Phe and Trp content, were extrapolated in such way and the results are depicted in Figure 3.

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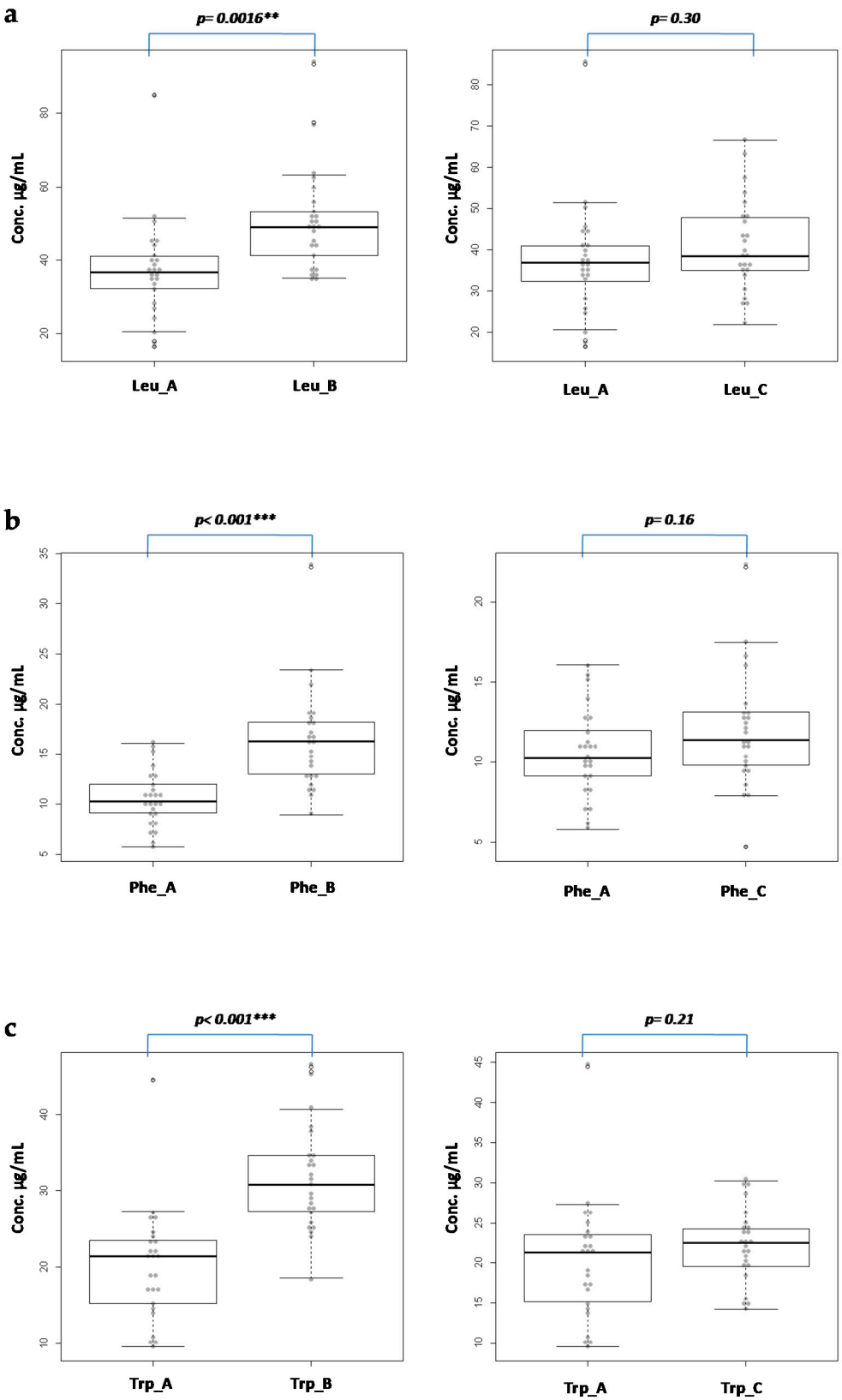

Figure 3. Beeswarm/box plots with ANOVA/TukeyHSD analyses of the Leu (a), Phe (b) and Trp (c) content on the three sampled groups (A-C).

The difference of the amino acid content between groups A and B is statistically significant. Indeed, the content level values of Phe and Trp from onions cultivated in Cannara compared with those produced in Imola are highly significant (group A vs B, ***p<0.001). Regarding the level of Leu,

the data are slightly less but still significant (group A vs B: **p= 0.002). Therefore, as a matter of fact, the geographical origin can influence in a statistically significant way the content level values of Leu, Phe, Trp.

From Figure 3, it is also clear that the irrigation mode does not affect the content of the selected amino acids: the difference in the content of the three selected amino acids are, indeed, not statistically significant ($p > 0.05$). This last part of the study strongly suggests a geographically-related content of the species under investigation.

3. Materials and Methods

3.1 Reagents

Pure water for HPLC analyses was obtained from a New Human Power I Scholar (Human Corporation, Seoul, Korea) purification system. All standard amino acids, as well as the eluent component acetonitrile (MeCN) and the ion-pair reagent heptafluorobutyric acid (HFBA), were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy).

3.2 Instrumentation

The HPLC analyses were carried out on a Shimadzu (Kyoto, Japan) Class Prominence equipped with two LC 20 AD pumps, an SPD M20A photodiode array detector, a CBM 20A system controller and a Rheodyne 7725i injector (Rheodyne, Cotati, CA, USA) with a 20 µL stainless steel loop.
A Varian 385-LC evaporative light scattering detector (ELSD) (Agilent Technologies, Santa Clara, CA, USA) was utilized for the HPLC analyses. The analog-to-digital conversion of the output signal from the ELSD was allowed by a common interface device. The adopted operative ELSD conditions for the analysis were: 50 °C nebulization temperature, 70 °C evaporation temperature, 2 L/min auxiliary gas flow rate (air) and 1 as the gain factor.

A Prevail C-18 (Phenomenex, Torrance, CA, USA), 250 mm × 4.6 mm i.d., 5 µm, was used as the analytical column. The column was conditioned with the selected mobile phase at a 1.0 mL/min flow rate for at least 40 min before use. All the analyses were carried out at a 1.0 mL/min flow rate. Column temperature was kept at 25 °C with a Grace (Sedriano, Italy) heather/chiller (Model 7956R) thermostat.

The Centrifuge Rotina 380 (Hettich, Tuttlingen, Germany) was employed for the extraction of amino acids from the freeze-dried onion samples.

3.3 Onion Sources

Group A: onion samples cultivated in Cannara (Province of Perugia, Umbria Region, Italy) and irrigated by water sprinkler method.

Group B: onion samples cultivated in Imola (Province of Bologna, Emilia Romagna Region, Italy) and irrigated by water sprinkler method.

Group C: onion samples cultivated in Cannara (Province of Perugia, Umbria Region, Italy) and irrigated by microirrigation method.

Irrespective of the provenience and with the due difference in terms of irrigation modality, all onions were cultivated in the same way and harvested in September 2013. All samples were provided by local farmers association, able to certify the cultivation characteristics and modalities.

All onion samples were managed and sampled by the 3A-Parco Tecnologico Agroalimentare dell'Umbria Società Consortile a r.l. (Todi, Italy).

3.4 Sample preparation and extraction of free amino acids

For each of the three groups A-C of onion Rossa da inverno sel. Rojo Duro, 25 bulbs were selected and manages. Each bulb was deprived of the outer drier, weighed, and chopped. The obtained mixture was subsequently freeze-dried and stored at 4 °C in sealed vials.

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The extraction of the amino acidic component from each freeze-dried sample was performed according to a protocol described in the literature [22] with some modifications. In particular, 20 mL distilled water were added to 1.0 g of freeze-dried onion sample. The obtained suspension was maintained under magnetic stirring for 3 min at 0 °C (ice bath), and centrifuged at 10000 rpm for 15 min. This operation was consecutively repeated three times by re-suspending the pellet every time. The final solution containing the amino acidic component was filtered under vacuum through a 0.45 µm nylon filter. Each obtained solution was lyophilized again and stored at 4 °C in sealed vials.

3.5 Amino acid separation and quantitation

Each extract was analyzed by using a previously developed IP-RP HPLC-ELSD methodology [15]. Samples were prepared at a concentration of 25 mg/mL, filtered through a nylon 0.45 µm filter and analyzed in triplicate.
The mobile phase gradient was obtained from eluent A (7 mM HFBA in pure water) and eluent B (net MeCN) as follows: 0-10 min 100% A, 10-30 min from 100 up to 75% A, 30-38 min from 75 up to 70% A, 38-39 min 100% A, 39-70 min 100% A.

3.6 Method Validation

The amino acid content in the onion samples was determined using a chromatographic external calibration method. For each of three amino acids of interest (Leu, Phe, Trp), four calibration solutions were prepared and run in triplicate. The average of the corresponding peak area values was employed to build-up the regression line.
The method was validated in terms of specificity, linearity, accuracy and precision, limit of detection (LOD) and Limit of quantification (LOQ). Precision and accuracy were estimated in both the short- (intra-day) and the long-term (inter-day) period.

3.6.1 Selectivity

Very appreciable separation (α) and resolution factor (R_S) values between the peaks of the three amino acids Leu, Phe, Trp were achieved in the selected experimental conditions. Moreover, no interference peaks were identified within the investigated analysis time.

3.6.2 Linearity

For each of the three amino acids of interest, calibration curves obtained after logarithm transformation of peak area and concentration values were used.
Log-log curves were always obtained with high R^2, and suitably used to appraise LOD and LOQ, as well as precision and accuracy of the method (Table 1).

3.6.3 LOD and LOQ

The LOD and LOQ values were calculated according to the following equations Eqs. (3) and (4):

$$C_{LOD}=3.3\frac{\sigma_y}{b} \quad (3)$$

$$C_{LOQ}=10\frac{\sigma_y}{b} \quad (4)$$

where C_{LOD} and C_{LOQ} are the sample concentrations corresponding to the LOD and LOQ, respectively, σ_y is the standard error of the corresponding regression, and b is the slope of the relative calibration equation (Table 1).

3.6.4 Intra-day and inter-day precision and accuracy

The method was validated for precision and accuracy, in both the short- (intra-day) and the long-term (inter-day) period.

The intra-day precision was assessed for each of the three investigated amino acids with the equations listed in Table 1. For all compounds, an external set of two control solutions, with concentration as indicated in Table 2, was run in triplicate. The procedure was repeated for a period of three consecutive days. The previously estimated mathematical models (Table 1) were then used to calculate the concentrations of the control solutions (observed concentrations, Table 2). The intra-day precision was evaluated as the relative standard deviation (RSD%) among the concentration values achieved from consecutive injections. For each control solution, the variation within replicate injections performed during a three-consecutive day period, and hence a total of nine injections, was used to calculate the inter-day precision (Table 3).

The percentage of the recovery, the so called "Recovery test" [20] was employed as test to estimate the accuracy of our IP-RP HPLC-ELSD method.

Similarly to the estimation of short and long-term precision, intra-day and inter-day accuracy were also determined with the same external solutions. Accordingly, while the former was determined by taking into account the three replicated runs for each control solution within a single day (Table 2), for the latter, the average value from nine determinations, along three days of analysis, was considered (Table 3).

3.7 Statistical methods

Boxplot and statistical analyses were performed with the aid of the open source software CRAN-R version 3.3.0. (http://www.R-project.org) [23]. In a classical boxplot the horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th-percentile, the whiskers indicate the highest and lowest values of the results, the outliers are displayed as circles. In the present study, the box plot representation was overlaid with a beeswarm plot.

A beeswarm plot is a 2D visualization technique where the experimental data points are plotted relatively to a fixed reference axis without the overlapping of the data points. It is useful to display the measured values for each data point and also the relative distribution of these values.

One-way ANOVA (Analysis of Variance) was used as a statistical test to assess the differences in means between the groups. Tukey's HSD (Honest Significant Difference) methodology, at confidence level of 95%, was further employed for multiple comparisons between all pair-wise means to determine how they differ [21]. $P < 0.001$ (***) values were considered high statistically significant.

4. Conclusions

In the present study, the content of amino acids extracted from the *Rossa da inverno sel. Rojo Duro* onion cultivar farmed and irrigated with two different methodologies in Cannara (Umbria Region, Italy) and in Imola (Emilia Romagna Region, Italy), was investigated.

A previously developed IP-RP HPLC method, coupled to a ELSD, was successfully applied for the direct analysis of the amino acids in onion extracts. Only the peaks corresponding to the three amino acids Leu, Phe, Trp, were not affected by matrix interfering peaks, and considered suitable for further quantifications and statistical evaluations. The high quality of the method, validated in terms of specificity, linearity, LOD and LOQ, accuracy and precision was demonstrated and revealed useful for the quantification of the three selected amino acids in the onion samples.

The statistical evaluation, based on the combination of the box plot representation with the beeswarm plot, indicated that the content of amino acids Leu, Phe, Trp was not affected by the irrigation mode, but was clearly influenced by the geographical origin of the onions (Cannara vs Imola).

Although further studies are needed to fully rationalize our results, these preliminary findings can represent a good starting point for considering the quantity of specific amino acids in the *Rossa da inverno sel. Rojo Duro* onion cultivar as a fingerprint of its geographical origin. Moreover, the developed approach can be applied to other onion cultivars/varieties thus contributing to their characterization, also limiting commercial frauds.

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Author Contributions: Roccaldo Sardella, Maura Marinozzi and Benedetto Natalini conceived and supervised the project; Federica Ianni and Antonella Lisanti performed the HPLC analyses, including validation; Emidio Camaioni and Lucia Pucciarini designed and performed the statistical study; Andrea Massoli and Luciano Concezi followed onion production, and provided the samples for the study; Roccaldo Sardella, Emidio Camaioni and Federica Ianni were involved in writing the manuscripts. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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Sample Availability: Samples of the data set used in the experiments are available from the authors.