Development and Validation of UPHLC-HRMS Methodology for the Determination of Flavonoids, Amino Acids and Organosulfur Compounds in Black Onion, a Novel Derived Product from Fresh Shallot Onions (*Allium* cepa var. *aggregatum*)

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ABSTRACT

Black onion, a new derived product from fresh onion, has been developed by processing (aging) fresh shallot onion in a temperature- and humidity-controlled room without using any artificial additives. The aim of this study was to adapt, optimize and validate two ultra-high-performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) methodologies for the determination of flavonoids, amino acids and organosulfur compounds in black onion. UHPLC-HRMS methods involving RP-C18 and HILIC columns were adapted and validated in terms of specificity, linearity, limit of detection (LD) and quantification (LQ), precision inter- and intra-day, recovery and matrix effect. Linearity ranged from 0.012-12.5 ngμL^{-1} and from 0.1-75 ngμL^{-1} for flavonoid and amino acids and organosulfur compounds, respectively. LD varied from 0.004-0.06 ngμL^{-1} and LQ from 0.012-0.2 ngμL^{-1}. The intra-day and inter-day precision for all compounds were less than 15% and the recovery ranged from 69 to 106%. The matrix effect ranged from 80 to 114% for flavonoids, amino acids and organosulfur compounds. The described methods were successfully applied for the correct separation and determination of 53 compounds in black onion. These results establish the value of these new two UHPLC-HRMS protocols in providing detailed compound profiles of black onion, highlighting their potential applicability to similar vegetables.

Keywords: black onion, flavonoids, amino acids, organosulfur compounds, HPLC-HRMS method validation

1. Introduction

Onion (*Allium cepa* L.) is one of the most important bulb crops and is commonly used as food, spice and medicinal plant almost worldwide. With an estimated global production rate of 5.7 Mt per year (http://faostat.fao.org, 2016), onion is the first most produced bulb crops in Spain, and together with garlic, is the most consumed bulb vegetable either fresh or after processing into various (cooked) products. Epidemiological and clinical studies have reported that the consumption of *Allium* vegetables such as onion, garlic and leek protects against the development of metabolic diseases such as diabetes (Akash, Rehman & Chen, 2014) or cardiovascular diseases (Bahadoran, Mirmiran, Momenan & Azizi, 2017). Besides, its consumption is associated with a reduce risk of developing diverse types of cancers including stomach, colorectal (Nicastro, Ross & Milner, 2015) and breast cancer (Pourzand et al., 2016), playing a significant role in human nutrition. From a nutritional point of view, free proteogenic amino acids account for 5-7% dry weight of an onion bulb with arginine and glutamine being the most abundant ones. In addition, onion is characterized by its high levels of health-promoting constituents comprising flavonoids and a huge variety of sulfur-containing compounds which accounted to its well-known nutritional properties (Böttcher, Krähmer, Stürtz, Widder & Schulz, 2017).

Nowadays, the food industry is looking for new foodstuffs with added value and functionality. Recently, a derived product from onion, black onion, is gaining great popularity among the Spanish consumers. This new product is made by processing (aging) fresh shallot onion in a temperature- and humidity-controlled room without using any artificial additives. This manufacturing process led to colour changes from white onion to black onion, and to improve organoleptic properties, also increasing the fruit-like sweetness of the final product. These changes are linked to substantial changes on chemical composition during the heating process of onion. However, the scientific data in relation to this issue is scare. Previous studies on an

analogous product called “black garlic” showed that the profiles of bioactives compounds in that product increased after the heating process of the raw garlic (Jung et al., 2014). Therefore, this new product not only has a great commercial value by its culinary use, but it could also be used as functional food.

To date, the analysis of the primary and secondary metabolites in foods is challenging due to their different structure, distributions and concentrations in plants vary greatly and the limitation of commercially available reference standards. Several techniques based on high-performance liquid chromatography coupled to mass spectrometry (LC-MS) offer versatile tools for addressing the identification and quantification of a wide number of compounds in onion samples. For instance, Soininen et al. (2014) analysed by LC-MS and NMR the composition of flavonols, free amino acids and organic acids in different Allium species. Other authors characterized the flavonol profile of several onion varieties by LC-DAD-ESI-MS-MS analysis (Bonaccorsi, Caristi, Gargiulli & Leuzzi, 2008) and identified a complete profiling of polar and semi-polar onion metabolites including fructooligosaccharides, proteinogenic amino acids, peptides, S-substituted cysteine conjugates, flavonoids and saponins by LC-ESI-QTOFMS (Böttcher et al., 2017). Moreover, an entire set of sulfur-containing onion metabolites in onion has been determined by RP-LC-ESI-Fourier transform ion cyclotron resonance mass spectrometry in conjunction with ^{13}C labelling (Nakabayashi et al., 2013).

To the best of our knowledge, no data on the fully characterization on primary (amino acids) and secondary metabolites (phenolic compounds and sulfur-containing compounds) of black onion have been reported. This study aims to identify and quantify these compounds in black onion, a new product derived from fresh shallot onion, by the optimization and validation of the extraction procedure as well as two rapid analytical UHPLC-HRMS methodologies.

2. Material and methods

2.1. Chemicals

Formic acid (FA), LC-MS grade acetonitrile, LC-MS grade methanol, ammonium acetate, ammonium formate, the reference compounds quercetin (95 %), rutin (94 %), isorhamnetin (99 %), quercetin-3-*O*-glucoside (98 %), kaempferol-3-*O*-rutinoside (98 %), luteolin (98 %), apigenin (95 %) and the amino acids leucine (98 %), isoleucine (98 %), phenylalanine (98 %), tryptophan (98 %), methionine (98 %), valine (98 %), proline (99 %), tyrosine (98 %), alanine (98 %), threonine (98 %), glycine (99 %), glutamic acid (99 %), glutamine (99 %), serine (99 %), asparagine (98 %), lysine (98 %), histidine (99 %), ornithine (99 %), aspartic acid (98 %), arginine (98 %) and gamma-aminobutyric acid (GABA) (98 %) and the organosulfur compounds alliin (95 %) and s-allyl-L-cysteine (SAC) (95 %) were purchased from Sigma-Aldrich (Madrid, Spain). All the standards used were not further purified. Deionized water was used throughout the analytical analyses.

2.2. Materials and sample preparation

Two kg of fresh shallot onions and black onions were obtained from a local supplier which provided authenticated fresh and black onion for the study. Both fresh and black onions were randomized and 0.5 kg was first frozen under liquid nitrogen to avoid enzymatic activity, then lyophilized and grinded afterwards to a final particle size of 10 µm using a mixer mill equipment (Retsch GmbH, Haan, Germany) and stored at -80ºC until analysis.

2.3. Extraction method

The optimization of the extraction of fresh and black onions were performed using three different solvents: deionized water:methanol (20:80, v/v) acidified with 1% formic acid (A), deionized water:methanol (50:50, v/v) acidified with 1% formic acid (B) and deionized water:acetonitrile (20:80, v/v) acidified with 1% formic acid (C). The extraction method was

as follow: 0.5 g of fresh or black onion lyophilized and grinded was mixed with 5 mL of solvent (A), (B) or (C) for 2 min at room temperature and the mixture was sonicated for 15 min and then centrifuged at 4900 rpm for 15 min. The supernatant was collected and residues were re-extracted twice using 5 mL of the same solvent by following the same protocol described previously. All the supernatants were pooled and frozen at -80 ºC until UHPLC-HRMS analysis.

2.4. UHPLC-HRMS Analysis

Identification and quantification of flavonoids, amino acids and organosulfur compounds in fresh and black onion extracts were carried out using an UHPLC-PDA-MS mass spectrometer system (Thermo Scientific, San José, CA, USA) comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 ºC (Dionex Ultimate 3000 RS, Thermo Corporation).

2.4.1. Analysis of flavonoids

Separation of flavonoids was performed on a 100 x 2.1 mm i.d. 1.8 µm Zorbax SB-C18 RRHD column (Agilent, Santa Clara, CA) preceded by a guard pre-column of the same stationary phase and maintained at 40 ºC. The mobile phases, A: acidified water 1% formic acid and B: acetonitrile, were pumped at a flow rate of 0.15 mL min^{-1} with a 33 min gradient starting in 3% B and maintained during 1 min, then rising 60% B in 24 min, maintained during 3 min and then rising 70% B in 5 min. After that, the column was equilibrated to the previous conditions within 5 min.

After passing through the flow cell of the PDA detector the column eluate went directly to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA) fitted with a Heated Electrospray Ionization Probe (HESI) operating in negative ionization mode for the determination of flavonoids. Full scans were recorder in m/z range from 100 to 1000 with a

148 resolution of 50.000 Hz and with a full AGC target of 100000 charges, using 2 microscans.
149 Analyses were also based on scans with in-source collision-induced dissociation (CID) at 25.0
150 eV. MS experiment condition with HESI in negative ionization mode was: (i) capillary
151 temperature was 275 ºC, the heater temperature was 100 ºC, the sheath gas was 19 units, the
152 auxiliary gas was 15 units, and the spray voltage was 4.0 kv.

153 Quality control samples (QC) were applied to assess and ensure the analytical process. The QC
154 samples, consisting of a pool of all fresh or black onion samples, were injected regularly
155 throughout the run. Data acquisition and processing were carried out using Xcalibur 3.0
156 software (Thermo Scientific, San José, CA).

157 *2.4.2. Analysis of amino acids and organosulfur compounds*

158 Separations of amino acids and organosulfur compounds in fresh and black onion extracts
159 were based on a 2.1 x 150 mm ACQUITY UPLC 1.7 µm BEH amide column (equipped with a
160 ACQUITY UPLC BEH amide 1.7 µm van-guard pre-column) (Waters, Spain) which was
161 maintained at 35 ºC and eluted using two mobile phases: A: deionized water with 5 mM of
162 ammonium acetate, 5 mM ammonium formate and 1% formic acid and B: acetonitrile, over the
163 course of 20 min at 0.4 mL min^{-1}. The gradient started with 5% of A rising 10% A in 0.5 min,
164 then rising 30% A in 8 min following 46% of A after 4.5 min and finally return to 5% A in 3
165 min and maintained during 4 min to equilibrate the column to the initial conditions.

166 After passing through the flow cell of the PDA detector the column eluate went directly to an
167 Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA) fitted with a Heated
168 Electrospray Ionization Probe (HESI) operating in positive ionization mode for the
169 determination of amino acids and organosulfur compounds. Full scans were recorder in m/z
170 range from 100 to 1000 with a resolution of 50.000 Hz and with a full AGC target of 100000
171 charges, using 2 microscans. Analyses were also based on scans with in-source

collision-induced dissociation (CID) at 25.0 eV. MS experiment condition with HESI in positive ionization mode was: (i) capillary temperature was 300 ºC, the heater temperature was 150 ºC, the sheath gas was 30 units, the auxiliary gas was 25 units, and the spray voltage was 3.5 kv. Quality control samples (QC) were also applied for the analysis of amino acids and organosulfur compounds as described previously.

2.4.3. Identification of flavonoids, amino acids and organosulfur compounds

Targeted identifications of phenolic compounds and amino acids and organosulfur compounds were achieved as follows: i) by comparing the exact mass and the retention time with available standards, ii) in the absence of standards, compounds were tentatively identified by comparing the theoretical exact mass of the molecular ion with the measured accurate mass of the molecular ion and searched against metabolite databases including Metlin, Phenol Explorer and more general chemical databases such as PubChem and ChemSpider. Metabolites having molecular masses within the pre-specified tolerance (≤ 5 ppm) of the query masses are retrieved from these databases. Quantification of phenolic compounds, amino acids and organosulfur compounds were carried out by selecting the theoretical exact mass of the molecular ion by reference to standard curves prepared in diluted fresh and black onion extracts. In absence of reference compounds, they were quantified by reference to the calibration curve of a closely related parent compound.

2.5. Method Validation

The method was fully validated for specificity, linearity, limit of detection (LOD) and quantification (LOQ), intra-day (repeatability) and inter-day precision and matrix effects, according to the FDA guidelines (FDA, 2015).

Linearity was assessed in reference compounds comprising 7 flavonoids, 21 amino acids and 2 organosulfur compounds by preparing individual stock solutions of all of them. Thus,

196 flavonoids were diluted in methanol, while amino acids and organosulfur compounds were
197 diluted in acidified deionized water (1% of FA). The stock solutions of flavonoids, amino
198 acids and organosulfur compounds were diluted and pooled to obtain standard solutions at a
199 final concentration of 200 µM of each compound. A total of eight working solutions with
200 concentrations ranging from 0.01 to 12.5 mgL^{-1} for flavonoids, and between 0.09 to 50 mg
201 L^{-1} and 0.1 to 75 mg L^{-1} for amino acids and organosulfur, respectively, were prepared.
202 Calibration curve were prepared using pure solvent (methanol or acidified deionized water)
203 and diluted matrices (fresh and black onion extracts), by triplicate, for matrix effect
204 evaluation.

205 *2.6. Statistical analysis*

206 Results are expressed as means ± standard deviations (SD) of three measurements for the
207 analytical determination. Multiple comparisons were carried out using one-way ANOVA,
208 followed by Tukey test. The level of significance was established at $p<0.05$. The statistical
209 software SPSS Statistic Program (v. 22) was used.

210

211 **3. Results and discussion**

212 *3.1. Optimization of the Extraction Method*

213 In this study, 5 flavonoids, 21 amino acids and 2 organosulfur compounds were selected to
214 perform the optimization of the extraction method from fresh and black onion, while 7
215 flavonoids, 21 amino acids and 2 organosulfur compounds were used for the validation study.
216 These compounds are commercially available and they were selected based on their previous
217 identification in onion samples (Juániz et al., 2016; Böttcher et al., 2017).

218 Recovery (%) of the selected compounds as representative components of flavonoids, amino
219 acids and organosulfur compounds in black and fresh onion using different extraction

solvents are shown in **Table 1**. The recovery rate was calculated using three different extraction solvents. These solvents are commonly used for the extraction of primary and secondary metabolites from food matrices (Nakabayashi et al., 2013; Sharma, Assefa, Ko, Lee & Park, 2015). For that, fresh and black onion samples were spiked with 10 µg all the standards and then were submitted to extraction in duplicate (before and after spiked) using three different solvents. Fresh and black onion samples were also submitted to extraction and either diluted and injected directly (blank samples) or spiked with a mixture of analytes (after-spiked). The recovery was calculated as the ratio between the areas of each analyte recorder in before-spiked samples minus the endogenous analytes in the matrix, divided by the area of each analyte recorder in after-spiked samples minus the endogenous analytes in the matrix, and expressed as percentage.

Thus, solvent A and B gave yields between 80.0 and 109.5% for flavonoids, showing no significant differences between these solvents used with the exception of rutin and isorhamnetin which had recovery rates less than 80% using solvent B in black onion matrix, (**Table 1**). In case of amino acids and organosulfur compounds, there were not significant differences using solvents A or B yielding recoveries between 80 and 111% in fresh onion matrix. However, some amino acids in black onion extraction using solvent B showed recovery values below 80%, such as tryptophan (73.4%), ornithine (74.5%) and methionine (79%) (**Table 1**). Recovery rates below 80% were found for an important number of tested compounds using solvent C. Based on these results, the subsequence steps for the method validation and quantification were done using as extraction solvent A which is a mixture of deionized water and methanol (20:80, v/v) acidified with 1% formic acid.

3.2. Development of UHPLC-HRMS Methods

The UHPLC-HRMS analytical method using HILIC column were developed and optimized to determine free amino acids and organosulfur compounds in fresh and black onion.

Modifications in the amount of ion-pairing reagent of the mobile phases (ammonium acetate and ammonium formate) and in the elution gradient, key factors for a good peak separation, were performed to obtain better peak resolution of underivatized amino acids in onion matrixes. A clear example of that is the better separation of the isomers leucine and isoleucine (**Figure 1)** achieved by using as mobile phase A: 100% acetonitrile, and as mobile phase B: 1% acidified deionized water with 5 mM ammonium acetate and 5 mM ammonium formate. The use of ammonium salts in the mobile phase also increase MS signal and peak shape, without affecting the sensitivity of the MS detector. In addition, the gradient was optimized to obtain the best resolution and the shortest run time. It is noteworthy that high-resolution MS (HRMS) used in this study could avoid the risk of inaccurate measurements caused by unresolved background interferences in complex matrices such as black onion.

3.3. Method Validation

3.3.1. Specificity, linearity, limit of detection and limit of quantification

Specificity was assessed as ppm deviation comparing mass error between the predicted m/z and observed m/z (FDA, 2015). As shown in Table S1, S2 and S3 (Supplementary Information), the ppm derivations obtained were < 5 ppm in all instances and are therefore considered as an acceptable level of mass accuracy. In addition, the retention time of each analyte was compared in blank solvent (methanol or distilled water) and in different matrices (fresh or black onion) previously spiked with standards. The relative standard deviation (RSD) was in all cases below 0.5% for 50 consecutive injections.

Linearity was assessed for 7 flavonoids, 21 amino acids and 2 organosulfur compounds and prepared in methanol/acidified water and in each matrix (fresh and black onion). Results of the linear regression analysis and the coefficient of determination (R^2) of flavonoids and amino acids and organosulfur compounds are shown in **Table 2** and **Table 3,** respectively**.**

Acceptable fitting was estimated by using the coefficient of determination (R^2). For all compounds, R^2 were above 0.983, showing acceptable linear relation between the range of concentration assayed and the detector response. Calibration curves were not force to pass through the origin. The limit of detection and limit of quantification of each compound in each matrix were determined by injecting consecutive dilutions of a working solution in either fresh onion extract or black onion extract until the S/N ratio of each compound showed a signal-to-noise (S/N) ratio ≥ 3 and ≥ 10, respectively. As shown in **Table 2**, the limits of detection in both onion matrices ranged from 0.004 to 0.007 ng μL^{-1} and the limits of quantification range from 0.012 to 0.024 ng μL^{-1} for flavonoids. Regarding amino acids, the limits of detection ranged from 0.01 to 0.06 ng μL^{-1} and the limits of quantification from 0.04 to 0.20 ng μL^{-1} (**Table 3**), in keeping with previously published data using HILIC coupled to MS analysis in wine, honey and apple juice (Gökmen, Serpen & Mogol, 2012) and in fruit juices (Guo et al., 2013). For organosulfur compounds, the limit of detection and quantification were 0.03 ng μL^{-1} and 0.1 ng μL^{-1}, respectively.

3.3.2. Intra- and inter-day precision

The intra-day precision (repeatability) was checked by measuring two different levels of concentration, one near the LOQ (L1) and other at higher concentration [5x LOQ (L2)] in diluted fresh and black onion extract and injected five times successively. The relative standard deviation for flavonoids in fresh shallot onion ranged from 0.7 to 3.7% for the level of concentration L1 (0.78 ngμL^{-1}) and from 1.5 to 3.8% for the level of concentration L2 (6.25 ngμL^{-1}) (**Table 2**), while in black onion matrix, the relative standard deviation (RSD) for flavonoids ranged from 0.4 to 1.5% for L1 (0.78 ngμL^{-1}) and from 0.5 to 2.7% for L2 (6.25 ngμL^{-1}) (**Table 2**). For amino acids, the RSD at concentration L1 (0.1 ng$\mu L^{-}1$) ranged from 0.7 to 12.1% and L2 (0.5 ng$\mu L^{-}1$) from 0.4 to 13.6% in fresh shallot onion while in black onion the RSD ranged from 0.9 to 13.8% and 1.0 to 10.1% at L1 and L2, respectively

294 (**Table 3**). The RSD for organosulfur compounds ranged from 1.5 to 5.2 % and 3.5 to 4.5 %
295 in fresh onion at concentration L1 and L2, respectively; while in black onion the RSD values
296 ranged from 2 to 3.8 % and 4.6 to 13.8 % for L1 and L2, respectively.

297 The inter-day precision was evaluated in five different days using the same procedure
298 described above for the intra-day precision. The values obtained in fresh onion for flavonoids
299 ranged from 1.9 to 13.1% for the level of concentration L1 and from 2.2 to 11.1% for the
300 level of concentration L2 (**Table 2**), while in black onion matrix, the values ranged from 4.8
301 to 9.5% for L1 and from 2.7 to 7.7% for L2 (**Table 2**). For amino acids, the RSD values
302 ranged from 2.0 to 15.2% at L1 and from 2.2 to 13.9% at L2 in fresh shallot onion, while in
303 black onion the RSD values ranged from 1.0 to 14.2% and 2.7 to 15.6% at L1 and L2,
304 respectively (**Table 3**). The RSD values for the inter-day precision for organosulfur
305 compounds ranged from 6.6 to 13.4 % and 5.7 to 11.4 % in fresh onion at concentration L1
306 and L2, respectively; while in black onion the RSD values ranged from 4.6 to 13.8 % and 2.5
307 to 7.3 % for L1 and L2, respectively. The results of the repeatability and precision of most
308 metabolites are in line with those proposed by the FDA (FDA, 2015) (RSD<15%).

309 *3.3.3. Matrix Effects*

310 Matrix effects (ME) were evaluated by comparing the slope of calibration curves prepared in
311 fresh and black onion extracts and the standard curves prepared in methanol with 1% FA for
312 flavonoids or acidified water for amino acids and organosulfur compounds, according to the
313 following equation:

$$ME = \frac{\text{Slope of calibration curve prepared in fresh or black onion}}{\text{Slope of calibration curve prepared in solvent}}\ x\ 100$$

314 Matrix effect variations are indicative of the susceptibility of the ESI source to matrix
315 composition and, as result, it is possible to observe ion suppression (values of matrix effect
316 less than 100%) or ion enhancement (values of matrix effects higher than 100%).

317 The matrix effect varied among the different analytes (**Table 2** and **3**). With respect to
318 flavonoids, it ranged from 93 to 110% in fresh onion and from 85 to 111% in black onion.
319 Amino acids and organosulfur compounds showed values of matrix effect ranged between 83
320 and 106% in fresh onion and between 80 and 114% in black ones. These values of matrix
321 effect determined for all compounds either in fresh or black onion matrices were less than 20%
322 and therefore were considered acceptable for the detection and further quantification of these
323 compounds by UHPLC-HRMS (Gasperotti, Masuero, Guella, Mattivi & Vrhovsek, 2014;
324 Feliciano, Mecha, Bronze & Rodríguez-Mateos, 2016).

325 *3.4. Identification and quantification of flavonoids, amino acids and organosulfur compounds*
326 *in fresh and black onion*

327 *3.4.1. Flavonoids*

328 A total of 10 flavonoids were identified in black or fresh onions through their mass
329 spectrometric characteristics and compared with data reported in literature. The basis of the
330 identification and the UHPLC-HRMS traces of flavonoids are shown in **Table S1**
331 (Supplementary Information), and are detailed as follows:

332 Peaks **1** and **2** has been identified as quercetin-7,4´-diglucoside and
333 quercetin-3,4´-diglucoside respectively based on their accurate masses at m/z 625.1410 and
334 taking into account previous reported data (Soininen et al., 2014; Fattorusso, Iorizzi, Lanzotti
335 & Taglialatela-Scafati, 2002).

336 Quercetin-3-*O*-glucoside and rutin (peaks **3** and **5,** respectively) were identified by its
337 retention time and MS characteristics in accordance with those of the authentic standards.
338 Additionally, peaks **8**, **9** and **10** were identified as quercetin, luteolin and isorhamnetin based
339 on their similarities of retention time and their MS characteristics with authentic standards.

340 The MS data also confirmed the presence of peak **4** at m/z 639.1570, consistent with

isorhamnetin-diglucoside. The identification of this compound as isorhamnetin-3,4´-*O*-diglucoside is in agreement with a previous work who studied the phenolic compound content of shallot onion (Bonaccorsi et al., 2008).

Further, the MS analyses confirmed the presence of quercetin and isorhamnetin hexosides (peaks **6** and **7,** respectively). These compounds, tentatively identified as quercetin-glucoside and isorhamnetin-glucoside, respectively, have been previously reported in shallot onion (Bonaccorsi et al., 2008; Fattorusso et al., 2002).

Table 4 summarizes the concentrations of the phenolic compounds in fresh and black onion. Free quercetin (144 µg g^{-1} FW) was the main flavonoid detected in black onion, representing 89.6% of the total flavonoids detected in black onion, with the remaining 10.4% consisting of four minor components such as quercetin-3-*O*-glucoside, quercetin-4-*O*-glucoside, luteolin and isorhamnetin. The major flavonoids in fresh onion were free quercetin (87 µg g^{-1} FW), quercetin-4-*O*-glucoside (39.4 µg g^{-1} FW) and two quercetin-diglucoside isomers (63 µg g^{-1} FW) which comprised 94.6% of the total flavonoids. Conversely, rutin, isorhamnetin-3,4´-diglucoside, quercetin-3-*O*-glucoside, isorhamnetin-4´-*O*-glucoside, luteolin and isorhamnetin were quantified as minor components. The values of free quercetin in fresh onion, although slightly lower, are in keeping with earlier studies who reported concentrations of quercetin in commercial onions ranging from 185 to 634 µg g^{-1} FW (Crozier, Lean, McDonald & Black, 1997) and 284 to 486 µg g^{-1} FW (Hertog, Hollman & Katan, 1992). This variation is due to differences in cultivars, maturity stages, origin places, harvest seasons or environmental conditions. Indeed, Fattorusso et al. 2002 reported the presence of high amounts of free quercetin and isorhamnetin and their glycosides: quercetin-4-glucoside, quercetin-diglucoside in shallot onion.

In general, the flavonoid content varied significantly among black (153.3 µg g^{-1} FW) and fresh shallot onion (199.7 µg g^{-1} FW), indicating potential flavonoid losses during the black

366 onion manufactured processes. Special attention should be given to individual compounds
367 such as free quercetin which is found in significantly higher quantity in black onion (144 µg
368 g^{-1} FW) compared with fresh onion (87 µg g^{-1} FW), probably from the thermal degradation of
369 quercetin-diglucosides present in fresh onions and which are not detected in black onion.

370 *3.4.2. Free Amino acids and organosulfur compounds*

371 A total of 21 free amino acids and 22 organosulfur compounds, including S-substituted
372 cysteine derivatives, were identified and quantified in fresh and black onions (**Table 5**). The
373 UHPLC-HRMS characteristics of each free amino acid and organosulfur compound are
374 shown in **Table S2 and S3** (Supplementary Information) together with their UHPLC-HRMS
375 traces (**Figure S2 and S3**, Supplementary Information).

376 The basis of the identification of amino acids was achieved by co-chromatography with
377 reference compounds and their fragmentation profiles upon low collision energy and by
378 reference to properties reported in previous related publications. Peaks **11** to **31** (**Table S2**,
379 Supplementary Information) corresponded to all the amino acids and were identified
380 compared with authentic standards. Further, peaks **32** to **53** (**Table S3**, Supplementary
381 Information), which corresponded to the organosulfur compounds, were identified in fresh
382 and black onion extracts on the basis of the data from previous related publications (Böttcher
383 et al., 2017; Nakabayashi et al., 2013; Kubec & Dadáková, 2009; Arnault et al., 2003).

384 Fresh onion showed significant higher concentrations of amino acids and organosulfur
385 compounds (3.60 mg g^{-1} FW and 2.54 mg g^{-1} FW, respectively) compared with that in black
386 onion (2.17 mg g^{-1} FW and 1.77 mg g^{-1} FW, respectively). Among them, arginine, glutamine,
387 glutamic acid, lysine, tyrosine, asparagine and leucine together with
388 γ-glutamil-*S*-(propenyl)cysteine sulfoxide and γ-glutamil-*S*-(1-propenyl)-cysteine were the
389 predominant amino acids and organosulfur compounds in fresh onion, in keeping with

previous published data (Kubec et al., 2009), while arginine, leucine, isoleucine, tyrosine, alanine and asparagine along with isoalliin are dominating in black onions. The abundance occurrence of isoalliin in black onion could be due to the enzymatic activity of the cysteine sulphoxidelyase activated during the heating process of fresh onions (Starkenmann, Niclass & Troccaz, 2011) and it could be a precursors of a wide range of sensory-active and health-beneficial compounds of black onion.

4. Conclusions

Two selective, sensitive, and precise UHPLC-HRMS methods were successfully optimized and validated to identify and quantify phenolic compounds, amino acids and organosulfur compounds in black and fresh onions, allowing the determination of 53 primary and secondary metabolites in both types. These methodologies are successful to analyse individual flavonoids, amino acids as well as organosulfur compounds in onion matrices showing good separation between compounds and highest limits of detection and quantification for the tested reference standards, without time consuming pre-treatment techniques involving complex extraction methods, clean-up steps and derivatization processes which leads to derivative instability, side reaction and reagent interferences prior to the analysis. Moreover, these results give a detailed profile of potential bioactive metabolites in black onion, a novel derived product from fresh onion, highlighting the large difference on the chemical composition between fresh and black onion due to the influence of the heating process involve in the production of black onion.

Abbreviations used

UHPLC-HRMS: ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry, FA: formic acid.

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Supporting information description

UHPLC-HRMS-based identifications and chromatograms of flavonoids, amino acids and organosulfur compounds.

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Figure Caption

Figure 1. Representative UHPLC-HILIC-HRMS chromatogram separation of leucine and isoleucine amino acids by using as organic mobile phase A) acetonitrile with 1 mM ammonium acetate and 1 mM ammonium formate or B) acetonitrile; and as aqueous mobile phase 1% formic acid with 5 mM ammonium acetate and 5 mM ammonium formate.

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531 **Table 1**. Recovery (%) of flavonoids, amino acids and organosulfur compounds from fresh
532 onion and black onion using three different extraction solvents. Solvent A: deionized
533 water:methanol (20:80, v/v) with 1% formic acid; solvent B: deionized water:methanol
534 (50:50, v/v) with 1% formic acid and solvent C: deionized water:acetonitrile (20:80, v/v) with
535 1% formic acid.

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	Compounds	Fresh Shallot Onion[a]			Black Onion[a]		
		Solvent A	Solvent B	Solvent C	Solvent A	Solvent B	Solvent C
Flavonoids	Quercetin	90.2a	91.7a	80.8a	109.5a	103.0a	80.0b
	Rutin	80.0a	83.7a	62.4b	83.7a	57.5b	64.1c
	Isorhamnetin	96.8a	86.5a	75.7a	88.2a	64.8b	52.7c
	Luteolin	83.9a	82.8a	76.8a	90.7a	108.6a	88.3a
	Apigenin	93.3a	81.7a	83.5a	94.2a	83.0a	94.3a
Amino Acids	Leucine	95.2a	97.6a	65.3b	101.3a	101.0a	65.5b
	Isoleucine	92.1a	96.1a	68.9b	110.6a	98.8b	46.9c
	Phenylalanine	96.6a	100.7a	67.3b	101.6a	103.0a	80.2b
	Tryptophan	89.1a	89.4a	55.4b	73.2a	73.4a	<20b
	Methionine	98.3a	90.0a	65.1b	78.2a	79.0a	<20
	Valine	93.8a	95.1a	66.6b	108.2a	98.7b	30.4c
	Proline	94.0a	93.2a	63.8b	107.2	98.0a	<20b
	Tyrosine	94.2a	97.4a	64.2b	103.7a	97.2a	<20b
	Alanine	89.7a	102.0a	77.5b	103.4a	98.3b	77.6c
	Threonine	94.9a	97.3a	65.5b	106.6a	99.2a	<20b
	Glycine	92.1a	92.2a	<20b	106.7a	98.6a	<20b
	Glutamic Acid	93.5a	96.7a	57.3b	105.8a	95.9b	<20c
	Glutamine	94.6a	97.4a	52.2b	104.8a	108.9a	<20b
	Serine	96.8a	98.1a	48.5b	104.5a	99.2b	<20c
	Asparagine	95.1a	96.3a	56.7b	103.1a	100.5b	<20c
	Lysine	99.7a	97.9a	<20b	105.0a	83.9b	<20c
	Histidine	110.9a	106.9a	<20b	105.9a	89.4b	<20c
	Ornithine	93.4a	80.0a	<20b	104.5a	74.5b	<20c
	Aspartic Acid	95.3a	96.3a	65.5b	100.6a	90.9a	<20b
	Arginine	97.1a	98.7a	<20b	103.3a	91.2b	<20c
	GABA	99.1a	96.7a	75.6b	107.7a	101.4b	84.7c
Organosulfur Compounds	Alliin	98.2a	99.0a	<20b	96.0a	103.0a	<20b
	SAC	97.1a	101.0a	<20b	70.8a	74.7a	40.0b

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538 [a] Different letters in a row denote significant differences ($p<0.05$) among the three solvents used
539 (one-way ANOVA followed by Tukey test).

Table 2. Summary of validation parameters for 7 flavonoids in fresh shallot onion and black onion.

Compounds	Linear Range (ng μL^{-1})	Slope	Intercept	R^{2a}	LOD (ng μL^{-1})[b]	LOQ (ng μL^{-1})[b]	Intra-day Precision[c]		Inter-day Precision[c]		Matrix Effects[d]
							L1	L2	L1	L2	
	Fresh Shallot Onion										
Quercetin	0.012-12.5	25402373	22555261	0.9923	0.004	0.012	1.7	2.5	8.5	5.5	93
Rutin	0.012-12.5	18055622	3113609	0.9975	0.004	0.012	1.2	2.5	1.9	2.2	98
Isorhamnetin	0.012-12.5	62416301	17835161	0.9942	0.004	0.012	1.3	1.6	9.3	5.6	110
Quercetin-3-*O*-glucoside	0.024-12.5	12372182	5040922	0.9909	0.007	0.024	0.7	1.5	7.6	7.1	98
Kaempferol-3-*O*-rutinoside	0.012-6.3	43785782	5867651	0.9927	0.004	0.012	0.8	3.2	5.5	5.8	104
Luteolin	0.012-12.5	53512956	5382539	0.9972	0.004	0.012	3.7	3.8	13.1	11.1	100
Apigenin	0.012-12.5	44384929	7064976	0.9838	0.004	0.012	1.0	1.8	8.5	4.6	106
	Black Onion										
Quercetin	0.012-6.3	30312758	7307594	0.9936	0.004	0.012	1.0	0.5	5.7	2.7	111
Rutin	0.012-12.5	16459260	1921621	0.9942	0.004	0.012	1.0	2.0	4.9	6.3	89
Isorhamnetin	0.012-12.5	55821170	13023749	0.9936	0.004	0.012	0.4	0.8	4.8	4.7	98
Quercetin-3-*O*-glucoside	0.012-12.5	10673405	81671	0.9985	0.004	0.012	1.0	0.5	8.1	4.1	85
Kaempferol-3-*O*-rutinoside	0.012-6.3	37877605	2631519	0.9960	0.004	0.012	0.8	1.0	5.5	5.8	90
Luteolin	0.012-12.5	49094166	4207330	0.9956	0.004	0.012	1.5	2.7	9.5	7.7	92
Apigenin	0.012-12.5	44197434	14094745	0.9907	0.004	0.012	0.7	0.8	7.4	5.4	106

[a]R^2: Coefficient of determination. [b]LOD: limit of detection. [b]LOQ: limit of quantification. [c]Intra- and inter- day precision correspond to RSD (%) of the injection of fresh and black onion extracts spiked with standards at a final concentration of L1 (0.78 ng μL^{-1}) and L2 (6.25 ng μL^{-1}). [d]Matrix effect is expressed as percentage.

549 **Table 3.** Summary of validation parameters for 21 amino acids and 2 organosulfur compounds in fresh shallot onion and black onion.

Peak	Compounds	Linear Range (ng μL^{-1})	Slope	Intercept	R^{2a}	LOD (ng μL^{-1})[b]	LOQ (ng μL^{-1})[b]	Intra-day Precision[c]		Inter-day Precision[c]		Matrix Effects[d]
								L1	L2	L1	L2	
	Amino acids	*Fresh Shallot Onion*										
11	Phenylalanine	0.10-50.0	1663641	691526	0.9980	0.03	0.10	2.6	2.5	13.9	8.7	105
12	Leucine	0.10-50.0	3245297	1434703	0.9971	0.03	0.10	1.7	2.4	5.6	8.2	83
13	Tryptophan	0.10-50.0	572486	-544643	0.9922	0.03	0.10	5.3	2.3	15.2	13.9	85
14	Isoleucine	0.10-50.0	4537671	1065457	0.9988	0.03	0.10	2.2	0.4	5.8	7.7	88
15	Methionine	0.10-50.0	1867736	-694741	0.9991	0.03	0.10	12.1	3.2	6.5	12.8	86
16	GABA	0.10-50.0	3175583	-953869	0.9993	0.03	0.10	6.9	2.9	11.0	9.2	92
17	Valine	0.10-50.0	1993399	1066633	0.9966	0.03	0.10	4.8	2.2	5.6	8.1	94
18	Proline	0.04-50.0	9080102	-530778	0.9997	0.01	0.04	11.5	3.0	11.4	12.9	106
19	Tyrosine	0.10-50.0	377503	-76068	0.9994	0.03	0.10	3.7	1.7	7.6	12.5	87
20	Alanine	0.10-50.0	1077476	-369036	0.9986	0.03	0.10	3.9	2.5	8.9	7.1	92
21	Threonine	0.10-50.0	820067	-158642	0.9996	0.03	0.10	2.9	0.6	4.3	7.9	93
22	Glycine	0.20-50.0	271977	-109487	0.9984	0.06	0.20	8.0	7.9	7.6	11.9	93
23	Glutamic acid	0.10-50.0	542528	303417	0.9991	0.03	0.10	1.9	2.1	5.5	10.0	87
24	Glutamine	0.10-50.0	496591	24948	0.9976	0.03	0.10	1.8	2.0	5.4	13.3	89
25	Serine	0.10-50.0	378872	99659	0.9981	0.03	0.10	1.9	2.6	5.3	9.9	91
26	Asparagine	0.10-50.0	605553	11650	0.9995	0.03	0.10	1.1	1.8	2.0	9.5	84
27	Aspartic acid	0.10-50.0	200135	-136497	0.9936	0.03	0.10	6.4	13.6	11.9	8.4	90
28	Arginine	0.10-50.0	1787725	326578	0.9995	0.03	0.10	0.7	1.5	6.8	7.2	106
29	Lysine	0.10-12.5	592527	-69029	0.9943	0.03	0.10	2.0	2.3	1.4	9.4	90
30	Ornithine	0.10-12.5	233713	-89535	0.9973	0.03	0.10	1.6	3.9	11.8	10.0	101
31	Histidine	0.10-25.0	536300	-675110	0.9900	0.03	0.10	6.1	0.7	1.8	2.2	88
	Organosulfur compounds											
36	Alliin	0.1-75.0	460250	-418104	0.9992	0.03	0.10	5.2	3.5	6.6	5.7	106
	SAC	0.1-75.0	966119	47217	0.9999	0.03	0.10	1.5	4.5	13.4	11.4	102

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	Amino acids	*Black Onion*										
11	Phenylalanine	0.10-50.0	1587579	1316871	0.9946	0.03	0.10	2.9	2.5	7.0	8.3	100
12	Leucine	0.10-12.5	3157625	490523	0.9980	0.03	0.10	1.2	2.0	12.1	6.6	81
13	Tryptophan	0.20-50.0	678836	-286383	0.9992	0.06	0.20	13.4	6.5	13.1	13.9	101
14	Isoleucine	0.10-50.0	4389002	1466016	0.9994	0.03	0.10	0.9	1.9	4.1	5.3	85
15	Methionine	0.10-50.0	1734599	-557069	0.9987	0.03	0.10	13.8	10.1	9.0	15.6	80
16	GABA	0.10-50.0	3264382	-1618886	0.9983	0.03	0.10	0.9	2.5	2.9	8.2	95
17	Valine	0.10-25.0	2348189	701542	0.9982	0.03	0.10	2.6	1.6	8.2	7.8	111
18	Proline	0.04-20.0	9510223	100806	0.9995	0.01	0.04	3.2	2.5	4.2	13.5	111
19	Tyrosine	0.10-50.0	348298	-17300	0.9999	0.03	0.10	1.3	1.2	9.0	10.3	80
20	Alanine	0.10-50.0	1205573	-458759	0.9985	0.03	0.10	1.9	1.5	6.7	7.9	103
21	Threonine	0.10-50.0	876703	-244956	0.9989	0.03	0.10	2.3	1.2	4.7	7.3	99
22	Glycine	0.10-50.0	261732	-84802	0.9991	0.03	0.10	3.5	1.1	4.7	6.8	89
23	Glutamic acid	0.10-50.0	544661	73654	0.9994	0.03	0.10	1.7	2.4	1.0	11.2	87
24	Glutamine	0.10-25.0	438271	-63229	0.9996	0.03	0.10	6.0	9.7	10.9	4.2	92
25	Serine	0.10-50.0	415478	36839	0.9993	0.03	0.10	1.7	1.4	3.3	9.3	100
26	Asparagine	0.10-50.0	671316	-46139	0.9994	0.03	0.10	2.8	1.0	6.1	8.6	94
27	Aspartic acid	0.10-50.0	226205	-145560	0.9929	0.03	0.10	3.0	8.5	14.2	5.8	102
28	Arginine	0.10-25.0	1487466	-832105	0.9922	0.03	0.10	2.3	1.0	10.9	8.0	88
29	Lysine	0.10-25.0	516008	-61100	0.9986	0.03	0.10	1.2	1.5	1.9	9.8	85
30	Ornithine	0.20-12.5	235670	-92026	0.9982	0.06	0.20	5.2	5.4	9.2	11.1	102
31	Histidine	0.20-25.0	586845	-1905888	0.9934	0.06	0.20	7.3	2.4	10.8	4.0	97
	Organosulfur compounds											
36	Alliin	0.10-10.0	427961	-169143	0.9972	0.03	0.10	3.8	4.6	2.5	2.7	95
	SAC	0.10-10.0	869774	-232084	0.9981	0.03	0.10	2.0	13.8	7.3	6.6	114

551 [a]R^2: Coefficient of determination. [b]LOD: limit of detection. [b]LOQ: limit of quantification. [c]Intra- and inter- day precision correspond to RSD (%) of the injection
552 of fresh and black onion extracts spiked with standards at a final concentration of L1 (0.1 ng μL^{-1}) and L2 (0.5 ng μL^{-1}). [d]Matrix effect is expressed as
553 percentage. [b] GABA: gamma aminobutyric acid

554 **Table 4**. Concentrations (µg g FW^{-1}) of individual flavonoids presented in fresh and black onion. Data
555 is expressed as mean values ± SDV (n=3).

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Peak	Compound	Fresh Shallot (µg g FW^{-1})	Black Onion (µg g FW^{-1})
1	Quercetin-7,4´-*O*-diglucoside	42.0 ± 4.0	nd
2	Quercetin-3,4´-*O*-diglucoside	21.0 ± 1.0	nd
3	Rutin	0.8 ± 0.1	nd
4	Isorhamnetin-3,4-diglucoside	1.7 ± 0.2	nd
5	Quercetin-3-*O*-glucoside	2.2 ± 0.3[a]	1.3 ± 0.1[b]
6	Quercetin-4-*O*-glucoside	39.0 ± 1.0[a]	6.4 ± 0.3[b]
7	Isorhamnetin-4´-*O*-glucoside	3.7 ± 0.3	nd
8	Quercetin	87.0 ± 6.0[b]	144.0 ± 2.0[a]
9	Luteolin	1.3 ± 0.2[a]	1.0 ± 0.1[a]
10	Isorhamnetin	1.0 ± 0.2[a]	0.6 ± 0.1[b]
	Total Flavonoids	**199.7 ±13.1** [a]	**153.3 ± 2.6** [b]

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558 [a] Different letters in a row denote significant difference ($p<0.05$) between fresh and
559 black onion. One-way ANOVA followed by Tukey test was performed to evaluate
560 significant differences ($p<0.05$).

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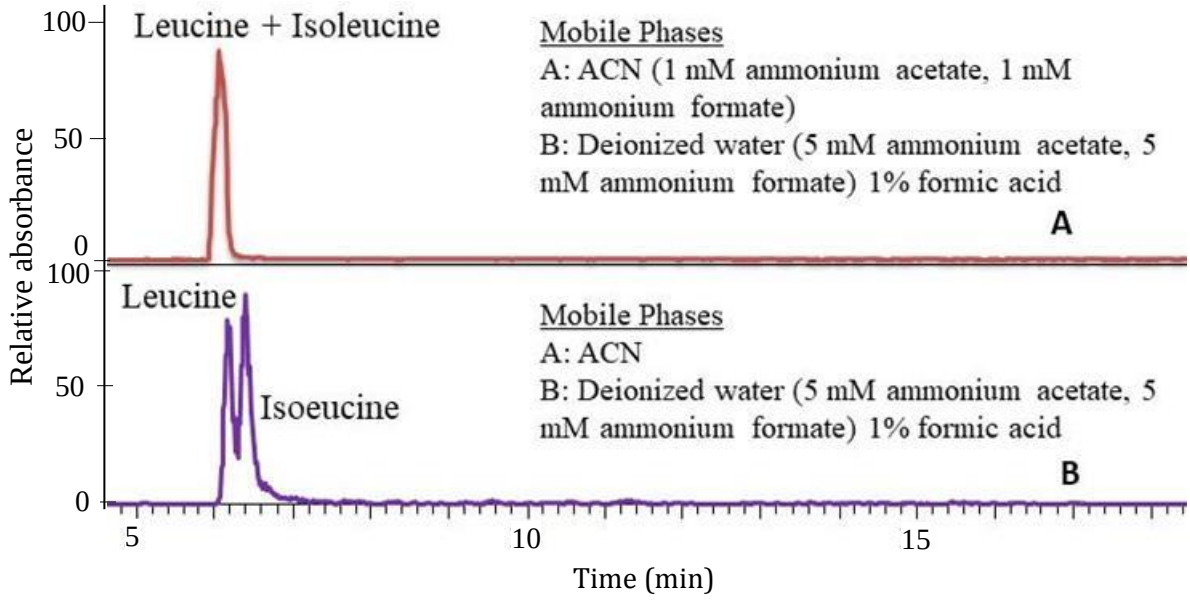
576 **Table 5**. Concentrations (µg g FW^{-1}) of individual amino acids and organosulfur compounds presented
577 in fresh shallot and black onion. Data is expressed as mean values ± SDV (n=3).

Peak	Compounds	Fresh Shallot (µg g FW-1)	Black Onion (µg g FW^{-1})
	Amino Acids		
11	Phenylalanine	28.4 ± 3.2[b]	85.0 ± 3.1[a]
12	Leucine	100.0 ± 11.0[b]	148.0 ± 5.3[a]
13	Tryptophan	87.1 ± 8.3[a]	2.2 ± 0.2[b]
14	Isoleucine	25.0 ± 2.1[b]	92.2 ± 10.0[a]
15	Methionine	7.3 ± 1.1	nd
16	GABA	15.4 ± 1.0[a]	10.1 ± 1.4[b]
17	Valine	53.2 ± 6.0[b]	83.4 ± 6.3[a]
18	Proline	17.1 ± 2.0[a]	12.9 ± 1.1[a]
19	Tyrosine	151.0 ± 16.2[b]	193.3 ± 13.2[a]
20	Alanine	28.4 ± 2.4[b]	179.7 ± 12.2[a]
21	Threonine	39.0 ± 4.0[b]	63.7 ± 5.1[a]
22	Glycine	12.2 ± 2.0[b]	88.8 ± 3.0[a]
23	Glutamic acid	313.1 ± 31.0[a]	106.3 ± 9.0[b]
24	Glutamine	504.4 ± 45.0[a]	3.1 ± 0.6[b]
25	Serine	28.9 ± 1.0[b]	49.3 ± 3.1[a]
26	Asparagine	331.0 ± 24.0[a]	142.0 ± 8.2[b]
27	Aspartic acid	50.0 ± 2.2[b]	102.0 ± 10.0[a]
28	Arginine	1299.1 ± 40.0[a]	629.9 ± 40.0[b]
29	Lysine	402.9 ± 31.0[a]	128.4 ± 8.2[b]
30	Ornithine	36.0 ± 3.3[a]	26.1 ± 1.1[b]
31	Histidine	75.0 ± 3.1[a]	24.0 ± 1.1[b]
	Total Amino Acids	**3604.5 ± 239.9[a]**	**2170.4 ± 142.2[b]**
	Organosulfur compounds		
32	*S*-(*S*-propyl)cysteine	6.6 ± 2.2[a]	5.5 ± 0.3[a]
33	*S*-(*S*-1-propenyl)cysteine	4.1 ± 0.2[a]	2.4 ± 0.1[b]
34	*S*-propyl-cysteine sulfoxide (Propiin)	9.6 ± 0.6[b]	12.2 ± 0.9[a]
35	*S*-(2-carboxypropyl)cysteine	4.2 ± 0.2[a]	2.9 ± 0.2[b]
36	*S*-(2-propenyl)cysteine sulfoxide (Alliin)	184.0 ± 20.1	nd
37	γ –Glutamyl-*S*-(*S*-propyl)cysteine-glycine	9.6 ± 0.6	nd
38	γ –Glutamyl-*S*-(*S*-1-propenyl)cysteine	4.1 ± 0.1[b]	6.2 ± 0.6[a]
39	γ –Glutamyl-*S*-(*S*-1-propenyl)cysteine-glycine	6.6 ± 0.5[a]	2.5 ± 0.1[b]
40	γ –Glutamyl-*S*-(propyl)cysteine	8.8 ± 0.8	nd
41	γ –Glutamyl-*S*-(1-propenyl)cysteine	311.1 ± 24.0	nd
42	*S*-(2-carboxypropyl)cysteine-glycine	102.3 ± 11.1[a]	3.2 ± 1.3[b]
43	(*S*-(*E*)-(1-propenyl)cysteine sulfoxide (Isoalliin)	131.0 ± 20.0[b]	1584.0 ± 66.4[a]
44	γ –Glutamyl-*S*-(*S*-methyl)cysteine-glycine	4.0 ± 0.2[a]	3.1 ± 0.2[b]
45	*S*-methyl-cysteine sulfoxide (Methiin)	29.2 ± 4.4[a]	5.6 ± 0.4[b]
46	*S*-methylcysteine (Deoxymethiin)	7.6 ± 0.6	nd
47	γ –Glutamyl-*S*-(2-carboxypropyl)cysteine-glycine	221.1 ± 21.3	nd
48	γ –Glutamyl-*S*-(2-carboxypropyl)cysteine glycine hexoside	3.6 ± 0.1	nd
49	γ –Glutamyl-*S*-propylcysteine sulfoxide	34.0 ± 5.0	nd
50	γ -Glutamyl-*S*-(1-propenyl)cysteine sulfoxide or γ -Glutamyl-*S*-(2-propenyl)cysteine sulfoxide	1387.2 ± 115.2[a]	120.1 ± 12.3[b]
51	3-Methyl-1,4-thiazane-5-carboxylic acid sulfoxide (Cycloalliin)	43.1 ± 4.0[a]	8.5 ± 1.1[b]
52	γ -Glutamyl-*S*-methylcysteine sulfoxide	14.4 ± 0.8[a]	7.9 ± 0.3[b]
53	γ -Glutamyl-*S*-methylcysteine	17.0 ± 0.8[a]	8.1 ± 0.6[b]
	Total Organosulfur Compounds	**2543.2 ± 232.8[a]**	**1772.2 ± 84.8[b]**

578 [a] Different letters in a row denote significant difference between fresh and black onion. One-way
579 ANOVA followed by Tukey test was performed to evaluate significant differences ($p<0.05$).

Figure 1


Leucine + Isoleucine
Mobile Phases
A: ACN (1 mM ammonium acetate, 1 mM ammonium formate)
B: Deionized water (5 mM ammonium acetate, 5 mM ammonium formate) 1% formic acid
A
Leucine
Isoeucine
Mobile Phases
A: ACN
B: Deionized water (5 mM ammonium acetate, 5 mM ammonium formate) 1% formic acid
B
Relative absorbance
100
50
0
5
10
15
Time (min)