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Glucosylated forms of serotonin and tryptophan in green coffee beans

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ABSTRACT

Green beans of Robusta and Arabica coffee species were examined by HPLC-ESI-MS^2 analysis for the content of glycosylated forms of serotonin and its *N*-methylated derivatives. The results showed for the first time the occurrence of serotonin 5-*O*-*β*-glucoside and tryptophan-N^1-glucoside in coffee beans. Both substances were identified by their product ion spectra and by comparison with synthesized authentic standards. Interestingly, serotonin 5-*O*-*β*-glucoside occurs at similar levels in beans of all varieties of Robusta and Arabica coffee species, whereas tryptophan-N^1-glucoside is present at high levels in beans of Robusta and at much lower levels in those of Arabica species. This striking difference in the content of tryptophan-N^1-glucoside could afford a new tool to discriminate between beans of Arabica and Robusta coffee species.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a tryptophan derived neurotransmitter involved in the regulation of important physiological functions in humans and animals such as mood, sleep and maintenance of energetic balance (Tecott, 2007; Voigt & Fink, 2015). The serotonin action starts with the binding to specific cell receptors able to modify cerebral and peripheral mechanisms involved in the control of food intake and satiety (Tecott, 2007; Voigt & Fink, 2015). It is well assessed that increased levels of serotonin in the central nervous system reduce feeling of appetite, for this reason the addition of biosynthetic serotonin precursors to diet is considered helpful in reducing food intake and promoting weight loss (Blundell & Leshem, 1975; Heisler, Chu, & Tecott, 1998; Latham & Blundell, 1979; Simansky, 1996; Voigt & Fink, 2015). Serotonin has been identified in numerous plant species including banana (Waalkes, Sjoerdsma, Creveling, Weissbach, & Udenfried, 1958), strawberry (Ly et al., 2008), rice (Kang, Kang, Lee, & Back, 2009), pineapple (West, 1960), where it exerts physiological roles in plant development and morphogenesis or in adaptation to environment (Kang et al., 2009; Ramakrishna, Giridhar, & Ravishankar, 2011). Serotonin and its derivative melatonin (*N*-acetyl-5-methoxytryptamine) have been also detected in green and roasted coffee beans of both Robusta (*Coffea canephora Pierre ex A. Froehner*) and Arabica (*Coffea arabica. L.*) species (Casal et al., 2004; Cirilo et al., 2003; Ramakrishna, Giridhar, Sankar, & Ravishankar, 2012) and thus the claim that extracts of green coffee beans may aid weight loss could find support in their serotonin content. In our previous studies, we reported the occurrence of serotonin, and all its *N*-methyl derivatives, 5-hydroxy-*N*-methyltryptamine, 5-hydroxy-*N*,*N*-dimethyltryptamine, and 5-hydroxy-*N*,*N*,*N*-trimethyltryptamine in leaves and other tissues of Citrus genus plants, where they probably exert a protective role against plant biotic stress (Servillo et al., 2013). Successively, again in Citrus plants, we also detected the 5-*O*-glucosylated forms of the above compounds (Servillo et al., 2015). Therefore, in the light of this evidence and considering that serotonin and its derivative melatonin are known to occur in coffee beans, we sought to evaluate whether *N*-methylated and glycosylated derivatives of serotonin may also be present in the coffee beans of Robusta and Arabica species.

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2. Materials and methods

2.1. Reagents

Tryptophan, 5-hydroxytryptamine (serotonin) hydrogenoxalate, cellobiose, β-glucosidase from almonds, formic acid and 1.0 g/kg solution of formic acid in water used for the LC-ESI-MS analyses were from Sigma-Aldrich (Milan, Italy). All other chemicals were of analytical reagent grade. Purified water was obtained from a Milli-Q system from Millipore (Milan, Italy).

2.2. Synthesis of serotonin 5-*O*-β-glucoside

Serotonin hydrogenoxalate (10 mg) and cellobiose (100 mg) were dissolved in 2.0 mL of 0.1 mol/L acetate buffer at pH 5.0. Then a solution of β-glucosidase from almonds in 0.1 mol/L sodium acetate buffer at pH 5.0 was added to a final enzyme concentration of 16.67 nkat/mL. The incubation was conducted at 37 °C. The time course of the transglycosylation reaction was monitored every 30 min by HPLC-ESI tandem mass spectrometry using the MS^2 transition 339.2 → 160. The quantitative evaluation of the produced serotonin 5-*O*-β-glucoside was achieved by analyzing the reaction mixture by HPLC with UV detection at 270 nm on a 25 cm Supelco Discovery C8 column eluted isocratically with 1.0 g/kg solution of formic acid in water. The area of the peak (r.t. 27.4 min) corresponding to serotonin 5-*O*-β-glucoside was compared with the peak area (r.t. 32.0 min) of a standard serotonin solution analyzed in the same manner assuming the same extinction coefficient for both substances.

2.3. Synthesis of tryptophan-N^1-glucoside

Tryptophan-N^1-glucoside was prepared according to the procedure of Gutsche, Grun, Scheutzow, and Herderich (1999) with some modification. Briefly, 500 mg of tryptophan and 900 mg of D-glucose were added with 10 mL of water and the pH adjusted to 2.0 with 1.0 mol/L HCl. The suspension was vortexed daily and left to stand 21 days at room temperature. The reaction time course was monitored every 4 days by HPLC-ESI tandem mass spectrometry using the MS^2 transition 367.2 → 205. The quantitative evaluation of the produced tryptophan-N^1-glucoside was achieved by analyzing the reaction mixture by HPLC with UV detection at 270 nm, on a 25 cm Supelco Discovery C8 column eluted isocratically with 1.0 g/kg solution of formic acid in water. The area of the peak corresponding to tryptophan-N^1-glucoside (r.t. 70.2 min) was compared with the peak area of a standard tryptophan solution (r.t. 80.5 min) analyzed in the same manner and utilizing, as molar extinction coefficients at 270 nm, 6000 $(mol/L)^{-1}$ cm^{-1} for tryptophan-N^1-glucoside (Gutsche et al., 1999) and 5200 $(mol/L)^{-1}$ cm^{-1} for tryptophan.

2.4. Extracts from pear purees

Fruits of Williams cultivar pear contain fair levels of tryptophan-N^1-glucoside (Diem, Bergmann, & Herderich, 2000), thus they were utilized as a natural source of this substance. Purees of Williams cultivar pear, purchased in a local market, were centrifuged at 18,000*g* for 30 min. The supernatant, after dilution 1:1 (v:v) with methanol, was allowed to stand 3 h at −20 °C and centrifuged again as before. The clear supernatant was desiccated in a rotavapor and the residue, dissolved in 1.0 g/kg formic acid in Milli-Q water, after filtration, was stored frozen at −20 °C until used for the determinations.

2.5. Coffee seed samples

Arabica and Robusta authentic monovarietal green coffee beans of certified geographical origin were provided by the Caffè Salimbene Company in Napoli (Italy). Seven Arabica coffee bean samples were from Ethiopia, Brazil, Colombia and Uganda and six Robusta coffee bean samples came from Uganda, India and Java. Four different lots of each sample were analyzed.

2.6. Coffee seed extracts

The seeds were initially washed with water, drained, and dried on filter paper. Then, they were finely grinded in a mortar and the powder homogenized in a mixer with 1.0 g/kg formic acid in the ratio 1:50 (w/w). Homogenates were kept under constant stirring for 3 h and then centrifuged at 18,000*g* for 30 min. The clarified extracts were filtered through 0.45 μm Millipore filters and stored frozen at −20 °C until used for the determinations. The powdered seeds were also extracted with mixtures of water containing 1.0 g/kg formic acid and methanol in the ratios 1:1 and 1:2 (v/v) in order to assess the efficacy of the various extraction mixtures.

2.7. HPLC-ESI-MS^2 analyses

Analyses were carried out using a HPLC Agilent 1100 series equipped with an auto-sampler coupled online with an Agilent LC-MSD SL quadrupole ion trap. The liquid chromatography separation of all serotonin derivatives was carried out on a Supelco Discovery-C8 column (250 × 3.0 mm, particle size 5 μm) eluted isocratically with 1.0 g/kg formic acid in water, at a constant flow rate of 0.1 mL/min. The injection volumes were 10 μL for each sample. Electrospray ionization (ESI) analysis was performed in positive ion mode, with nitrogen as the nebulizing and drying gas under the following conditions: nebulizer pressure, 206,843 Pa; drying temperature, 350 °C and drying gas, 7 L/min. The ion charge control (ICC) was applied with the target set a 30,000 and maximum accumulation time at 20 ms. Multiple reaction monitoring (MRM) was used for the detection of analytes in HPLC-ESI-MS^2 analyses. The retention times and peak areas of the monitored fragment ions were determined by the Agilent software Chemstation version 4.2.

2.8. Statistical analysis

Statistical analysis was performed using the XLSTAT software, version 2012 (Addinsoft, Paris, France). The variability of tryptophan-N^1-glucoside and serotonin 5-*O*-β-glucoside concentrations between Arabica and Robusta groups and within-group were examined with analysis of variance (ANOVA). Categorical data were analyzed with the Fisher exact test (2-sided). A p value less than 0.05 was considered statistically significant. All tests were two-tailed. Data are presented as means ± SD.

3. Results and discussion

Our recent reports of the presence of serotonin, all its *N*-methylated derivatives and their glucosylated forms in several Citrus genus plants (Servillo et al., 2013; 2015), prompted us to investigate the possibility that those compounds might also be present in coffee beans where serotonin and its derivative melatonin occur (Casal et al., 2004; Ramakrishna et al., 2012). To this aim, we subjected various extracts of green coffee beans to HPLC-ESI tandem mass analysis. The extraction procedure employed only water with 1.0 g/kg formic acid as it gave better results than those obtained employing mixtures of water with 1.0 g/kg formic acid and methanol (data not shown). A representative HPLC-ESI-MS^2

chromatogram of an extract of *Coffea canephora Pierre ex A. Froehner* (Robusta) seeds from Uganda is shown in Fig. 1. The chromatogram was obtained in multiple reaction monitoring mode (MRM) by isolating the ion masses at *m/z* 177.1, 191.1, 205.1, 219.1, 339.2, 353.2, 367.2 and 381.2 which coincide with those of the protonated masses of serotonin, *N*-methylserotonin, *N,N*-dimethylserotonin, *N,N,N*-trimethylserotonin, and their glycosylated forms, respectively. In the chromatogram only three peaks appear. The first peak, emerging at retention time of 27.4 min, was detected by isolating at *m/z* 339.2 and shows the MS^2 fragmentation pattern reported in Fig. 1 (**panel A**). Both retention time and MS^2 fragmentation pattern of peak 1 coincide with that of serotonin 5-*O*-*β*-glucoside that we previously isolated in Citrus plants and characterized for its mass spectrometric features (Servillo et al., 2015). Moreover, we also synthesized serotonin 5-*O*-*β*-glucoside and confirmed the identity of the peak 1 compound as serotonin 5-*O*-*β*-glucoside from comparison with the synthesized standard (data not shown). The second peak, emerging at retention time of 32.0 min, was detected by isolating at *m/z* 177.1. Fig. 1 (**panel B**) shows its MS^2 fragmentation pattern. This peak corresponds to serotonin, as confirmed by comparison of retention time and MS^2 fragmentation pattern with that of the authentic standard.

The third peak, emerging at retention time of 70.2 min, was detected by isolating at *m/z* 367.2. Fig. 1 **(panel C)** shows its MS^2 fragmentation pattern. However, although the isolation at *m/z* 367.2 coincides with that of *N,N*-dimethylserotonin glucoside, both retention time and MS^2 fragmentation pattern allow to exclude with certainty that peak 3 corresponds to this compound. Actually, our previous studies dealt with the chromatographic and mass spectrometric features of all serotonin *N*-methylated and 5-*O*-glucosylated derivatives (Servillo et al., 2015). In particular, we reported the MS^2 fragmentation of *N,N*-dimethylserotonin-5-*O*-glucoside, which indeed is very different from that of peak 3. Furthermore, in the same chromatographic conditions, the peak of *N,N*-dimethylserotonin-5-*O*-glucoside elutes at r. t. of 40.4 min, whereas peak 3 of the unidentified compound elutes at r. t. of 70.2 min.

3.1. Identification of tryptophan-N^1-glucoside in coffee beans

The identity of the unknown substance of peak 3 was disclosed from the analysis of its MS^2 fragmentation pattern where the two most abundant fragments were at *m/z* 205 and *m/z* 188 (Fig. 1, **panel C**), which gave a strong clue of the occurrence of a tryptophan derived moiety in the molecular structure of the unknown compound. In fact, *m/z* 205 could correspond to the formation in the course of the MS^2 fragmentation process of protonated tryptophan which, in turn, is well known to easily give an abundant fragment at *m/z* 188 by ammonia neutral loss (Dookeran, Yalcin, & Harrison, 1996). Therefore, we hypothesized that the unknown compound was a glycosylated derivative of tryptophan. Indeed, a tryptophan glucoside, in which *N*-glycosidic linkage of hexose moiety to the indole nitrogen is present, was discovered in some fruit species, and NMR and mass spectrometric studies identified the substance as N^1-(*β*-D-glucopyranosyl)-L-tryptophan (Diem et al., 2000; Gutsche et al., 1999). As the substance was found particularly abundant in fruits of Williams cultivar pear, we first compared the HPLC-ESI-MS^2 chromatograms of Robusta coffee beans extracts with those of extracts of Williams pear fruit puree and, successively, compared the HPLC-ESI-MS^2 chromatograms of Robusta coffee beans extracts with that of authentic N^1-(β-D-glucopyranosyl)-L-tryptophan synthesized as described (Diem et al., 2000). In both comparisons, a perfect identity with retention time and MS^2 fragmentation pattern of peak 3, reported in Fig. 1, **panel C**, was observed, thus confirming that the unknown compound was N^1-(β-D-glucopyranosyl)-L-tryptophan.

3.2. Distribution tryptophan-N^1-*β*-glucoside and serotonin 5-*O*-*β*-glucoside in green coffee beans

Quantification of tryptophan-N^1-*β*-glucoside and serotonin 5-*O*-*β*-glucoside was conducted on green coffee beans of different origins, six of which belonged to Robusta and seven to Arabica species (Fig. 2, **panel A, B**). The analysis was performed by HPLC-ESI-MS^2, as described in experimental section. It is evident the striking difference of tryptophan-N^1-glucoside content between Robusta and Arabica species (Fig. 2). In fact, in Arabica samples the concentration was below 1.0 mg/kg (mean 0.8 ± 0.2 mg/kg), whereas in Robusta samples it was in the range 40–50 mg/kg (mean 45.4 ± 5.2 mg/kg), with a mean substance content of about 50 times greater in Robusta than in Arabica species. The converse holds for serotonin 5-*O*-*β*-glucoside, although in much less marked manner. In fact, contrary to that observed for tryptophan-N^1-glucoside, the levels of serotonin 5-*O*-*β*-glucoside were lower (about one half) in Robusta (3.1 ± 0.4 mg/kg) than in Arabica species (5.4 ± 0.8 mg/kg) (Fig. 2, **panel B**). The difference in the contents of tryptophan-N^1-glucoside and serotonin 5-*O*-β-glucoside between Robusta and Arabica groups were statistically significant although much more for the content of tryptophan-N^1-glucoside ($p < 0.0001$) than that of serotonin 5-*O*-*β*-glucoside ($p < 0.010$).

Extracts of several blends of toasted coffee seeds were also analyzed as described above. However, both tryptophan-N^1-glucoside and serotonin 5-*O*-*β*-glucoside were not detected in any of them, thus indicating that the roasting process leads to their decomposition.

The noticeable difference of tryptophan-N^1-glucoside levels between Robusta and Arabica green seeds, could make this substance a suitable marker in the quality control for authentication of the botanical origin of batches of green coffee beans for industrial processing. As matter of fact, Arabica is much more appreciated than Robusta species for its organoleptic qualities and has a significantly higher market value. Therefore, the determination of the authenticity of seeds intended for the subsequent roasting process is an aspect of primary importance both for product quality and economic reasons. The evaluation of the quality of green coffee bought by companies for further processing, although still based on visual inspection, tends to move towards more objective determination of parameters as odor, color, shape, hardness, and, last but not least, chemical composition of seeds, aimed to assess their botanical origin (Feria-Morales, 2002; Toci, Farah, Pezza, & Pezza, 2016). Currently, the fraudulent addition of Robusta beans in batches marketed as 100% of green or roasted Arabica beans is detected by measuring the concentration of 16-*O*-methylcafestol (16-OMC), a specific pentacyclic diterpene alcohol derived from cafestol. This substance, identified and isolated from Robusta beans (Speer & Mischnick-Lubbecke, 1989; Speer, Tewis, & Montag, 1991), is absent in Arabica beans (Frega, Bocci, & Lercker, 1994; Trouche, Derbesy, & Estienne, 1997). Moreover, being stable during the roasting process, it has been proposed as an appropriate marker for assessing the presence of Robusta species in blends of roasted coffee powder (Speer & Kolling Speer, 2006; Speer et al., 1991). On the basis on these studies, a HPLC method for 16-OMC determination in roasted coffee product has been validated (DIN method No. 10779, 1999). However, this method requires a somewhat long process (about 7–8 h) for sample preparation before analysis. Recently, as an alternative to HPLC determination of 16-OMC, a new method, based on high-resolution proton NMR spectroscopy, has been proposed (Schievano, Finotello, De Angelis, Mammi, & Navarini, 2014). This method is able to detect the level of Robusta in Arabica beans at percentage of about 1%, through the

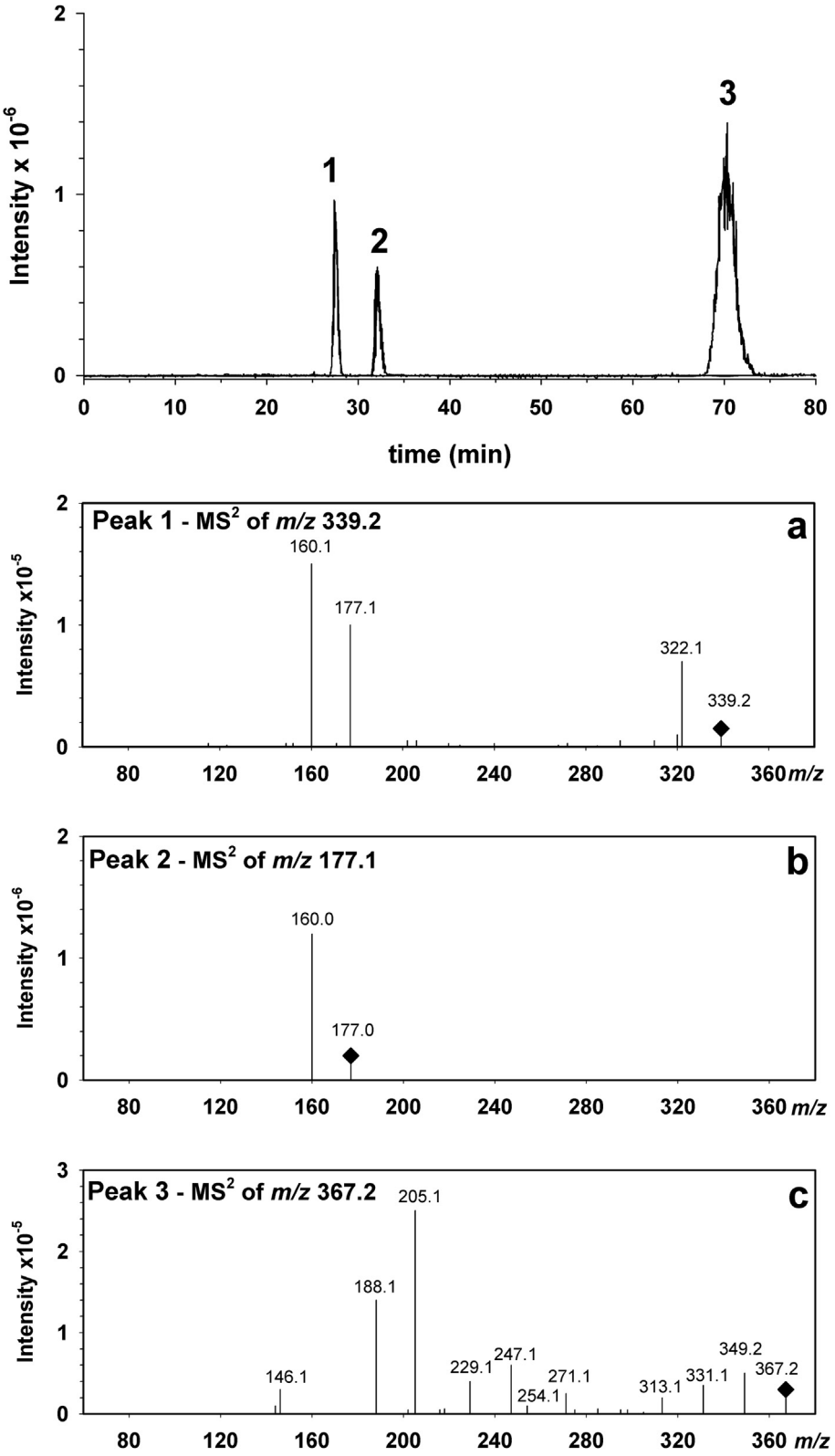

Fig. 1. Representative MS^2 extracted ion chromatogram, in MRM mode, of an aqueous extract of *Coffea canephora Pierre ex A. Froehner* (Robusta) green seeds from Uganda. Peak 1, at retention time of 27.4 min, corresponds to serotonin 5-*O*-*β*-glucoside. The MS^2 transition followed was 339.2 → 322, the MS^2 fragmentation pattern is reported in Panel A. Peak 2, at retention time of 32.0 min, corresponds to serotonin. The MS^2 transition followed was 177.1 → 160, the MS^2 fragmentation pattern is reported in Panel B. Peak 3, at retention time of 70.2 min, corresponds to tryptophan-N^1-glucoside. The MS^2 transition followed was 367.2 → 205, the MS^2 fragmentation pattern is reported in Panel C.

quantitative determination of the free and esterified 16-OMC directly in seed extracts without further purification process. However, the method requires expensive instrumentation hardly available in quality control laboratories.

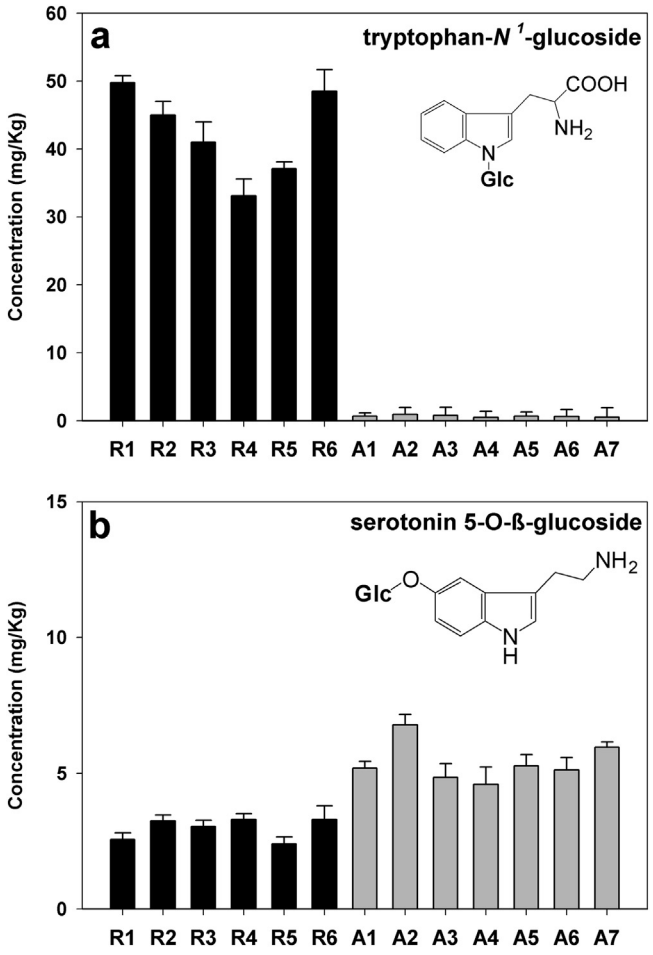

Fig. 2. Relative levels of tryptophan-N^1-glucoside and serotonin 5-*O*-β-glucoside in green seeds of Robusta and Arabica coffee species of different origins. The mean concentration of tryptophan-N^1-glucoside was 0.8 ± 0.2 mg/kg for Arabica and 45.4 ± 5.2 mg/kg for Robusta samples ($p < 00001$). In the same samples, the mean concentration of serotonin 5-*O*-β-glucoside was 3.1 ± 0.4 mg/kg for Robusta and 5.4 ± 0.8 mg/kg for Arabica species ($p < 0.010$). The level of each compound is the average of four determinations. Bars represent the standard deviations.

3.3. Determination of composition of green coffee seed blends from the tryptophan-N^1-glucoside content

On the basis of the above results, a possible simple way to quantify the addition of Robusta to Arabica seeds is devised. This can be accomplished by analyzing with the described HPLC-ESI-MS^2 procedure the same volumes of pure Robusta and Arabica seed extracts and that of the unknown blend of seeds. As matter of fact, we prepared and then analyzed several blends, at different ratios of Robusta and Arabica green seeds, obtained by mixing in the due amounts both types of seeds. Successively, 5.0 g of pure Arabica seeds, 5.0 g of pure Robusta seeds and 5.0 g of the prepared blends were extracted as described before, and the same volumes (10 μl) of each extract was analyzed by HPLC-ESI-MS^2. Finally, the areas of the peaks corresponding to tryptophan-N^1-glucoside were determined from each chromatogram.

Say **A**, **R** and **U** the area of the peaks corresponding to tryptophan-N^1-glucoside in the chromatogram of Arabica, Robusta and Unknown blend of seeds, respectively. Assuming that the contents of tryptophan-N^1-glucoside in Robusta and Arabica seeds used as standards are the same of those of Robusta and Arabica seeds in the unknown blend, the fraction (X_1) of Robusta seeds in the unknown blend in which the fraction of Arabica seeds is X_2, is given by: $X_1 = (\mathbf{U}-\mathbf{A})/(\mathbf{R}-\mathbf{A})$.

In fact: $\mathbf{U} = X_1 \cdot \mathbf{R} + X_2 \cdot \mathbf{A}$ where $X_1 + X_2 = 1$, then

$$U = X_1 \cdot R + (1 - X_1) \cdot A$$

and solving for X_1, we get $X_1 = (\mathbf{U}-\mathbf{A})/(\mathbf{R}-\mathbf{A})$.

Obviously, the higher the difference ($\mathbf{R}-\mathbf{A}$) the more the method is sensitive. In our case, we found that the content of tryptophan-N^1-glucoside in Robusta seeds is meanly 50 times than that of Arabica seeds. Therefore, as peak areas are proportional to concentrations, we also have that.

$\mathbf{R} = 50 \cdot \mathbf{A}$. This means, applying the above equation, that a blend containing 5% of Robusta seeds ($X_1 = 0.05$) has the area **U** which is 3.45 times greater than the area **A**. The result was still satisfactory with the blend in which Robusta seeds were as low as 2% of the total seeds. In facts, in this case, as expected, the area **U** we observed was about twice of area **A**.

The above results show that the mass spectrometric determination of tryptophan-N^1-glucoside could be advantageously employed to detect the authenticity of coffee Arabica seed with contamination of Robusta seeds above 2%. Furthermore, the procedure for sample preparation is relatively simple and quantification is accomplished with mass spectrometric technique that offers excellent selectivity and employs an instrumental apparatus frequently present nowadays also in quality control laboratories of small companies.

4. Conclusions

The most used coffee species in the preparation of commercial coffee blends are represented by *Coffea canephora Pierre ex A. Froehner* (Robusta) and *Coffea arabica. L.*, but the latter is most appreciated by the consumers for lower caffeine content and superior flavor than Robusta, thus accounting for about 70% of the world production. However, the Robusta species is less demanding in terms of cultivation and more productive and resistant to diseases. For these reasons, the Arabica species has a consistently higher commercial value than Robusta. In the era of market globalization, adulteration of food and beverage has become a major issue, involving many edible products, including coffee. Therefore, the assessment of product authenticity is of prime importance for economical and quality reasons. As for coffee, various more or less specific components, such as sterols, terpenoids, amino acids, metals and volatile compounds, have been proposed to discriminate among the coffee species and mainly utilized as indicators of Robusta beans in commercial coffee blends. The data reported in this paper show for the first time the occurrence in green coffee beans of the two metabolites serotonin 5-*O*-β-glucoside and tryptophan-N^1-glucoside. Besides, the findings that the levels of the latter are much higher in Robusta than in Arabica species could afford a possible new tool for detection of coffee beans of the Robusta species with rapid, specific, and sensitive mass spectrometric measurements requiring minimal sample manipulation and instrumentations which are currently within the reach also of small quality control laboratories.

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