Sugars Profiles of Different Chestnut (*Castanea sativa* Mill.) and Almond (*Prunus dulcis*) Cultivars by HPLC-RI

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Abstract Sugar profiles of different almond and chestnut cultivars were obtained by high-performance liquid chromatography (HPLC), by means of a refractive index (RI) detector. A solid-liquid extraction procedure was used in defatted and dried samples. The chromatographic separation was achieved using a Eurospher 100-5 NH_2 column using an isocratic elution with acetonitrile/water (70:30, *v*/*v*) at a flow rate of 1.0 ml/min. All the compounds were separated in 16 min. The method was optimized and proved to be reproducible and accurate. Generally, more than 95% of sugars were identified for both matrixes. Sugars profiles were quite homogeneous for almond cultivars; sucrose was the main sugar (11.46±0.14 in *Marcona* to 22.23±0.59 in *Ferragnes* g/100 g of dried weight), followed by raffinose (0.71±0.05 in *Ferraduel* to 2.11±0.29 in *Duro Italiano*), glucose (0.42±0.12 in *Pegarinhos two seeded* to 1.47±0.19 in *Ferragnes*) and fructose (0.11±0.02 in *Pegarinhos two seeded* to 0.59±0.05 in *Gloriette*). Commercial cultivars proved to have higher sucrose contents, except in the case of *Marcona*. Nevertheless, chestnut cultivars revealed a high heterogeneity. Sucrose was the main sugar in *Aveleira* (22.05±1.48), *Judia* (23.30±0.83) and *Longal* (9.56±0.91), while glucose was slightly prevalent in *Boa Ventura* (6.63±0.49). The observed variance could serve for inter-cultivar discrimination.

Keywords *Castanea sativa* Mill. · HPLC-RI · *Prunus dulcis* · Sugars profiles

Abbreviations

FAO	Food and Agriculture Organization
HPLC	High-performance liquid chromatography
IS	Internal standard
PDO	Protected designation of origin
RI	Refractive index

Introduction

Edible nuts, from which almond (*Prunus dulcis*) and chestnut (*Castanea sativa* Mill.) are typical examples, are cultivated in a variety of growing conditions and climates, being globally popular and valued for their sensory, nutritional, and health attributes [1]. Recently, almonds are among fruits that are considered important for human health [2]. Almond tree, the number one nut tree produced on a global basis, is especially spread through and well adapted to the whole Mediterranean region, from which about 28% of the world production is obtained. In Portugal, almond is a traditional crop, mainly spread through Algarve and Baixo Alentejo in the south, and "Terra Quente Transmontana" in the north [1], with 24,522 crops representing 36,530 ha [3]. In fact, almonds are readily accepted worldwide and are therefore used in a variety of food products. Incorporating almonds in food

products adds value to that product in a variety of ways [4].

According to the FAO, chestnut worldwide production is estimated in 1.1 million tons. Europe is responsible for about 12% of global production, with relevance for Italy and Portugal, corresponding to 4% and 3%, respectively. Trás-os-Montes region represents 75.8% of Portuguese chestnut crops and 84.9% of chestnut orchards area (23,338 ha). In 1994, three protected designations of origin (PDO) called "*Castanha da Terra Fria*", "*Castanha dos Soutos da Lapa*" and "*Castanha da Padrela*" were created [5]. In fact, chestnut is one of the most important cultivated fruits in Portugal, where it has a relevant place at the socioeconomic level, reaching an annual production of more than 30,000 tons [6]. Due to its commercial potential, Portuguese government has been granting financial support for the reinforcement of chestnut and almond production. Hence, the assessment of the commercial quality of these fruits is an essential activity. Actually, contents of protein, oil, free sugars and other components, affect industrial use of almond kernel [2].

Carbohydrates are relevant components in chestnut and almond, especially starch, which is followed by sucrose. This disaccharide is one of the most important parameters in the assessment of fruit quality, once sugar content and composition can be lowered or modified by several conditions, like storage temperature, relative humidity, harvest time, oxygen level or packaging [7]. The free sugar composition can also be influenced by different varieties, genotypes, ecological conditions, or technical and cultural practices. Together with sucrose, glucose, fructose and raffinose are present in significant amounts and may contribute for the identification of a specific chestnut or almond cultivar. [2].

In view of the ongoing reinforcement of the chestnut and almond orchards area in Northeastern Portugal, the quality evaluation of these fruits represents an important task. Furthermore, the enhancement of nuts nutritional quality through cultural practices may improve their use in a global basis, since they are globally accepted as high-quality fruits. Furthermore, our research group has been interested in the chemical characterization [5, 8] and bioactive properties [1, 9] of these fruits. Although almonds and chestnuts are worldwide popular fruits, studies characterizing their sugar composition are limited. Once that sugar composition is one of the most important parameters in the assessment of commercial quality of a determined fruit, this parameter was analyzed in different almond and chestnut cultivars using a HPLC methodology completely validated for these analyses.

Materials and Methods

Standards and Reagents

Acetonitrile 99.9% was of HPLC grade from Lab-Scan (Lisbon, Portugal). Ethanol and petroleum ether were of analytical grade purity and were also supplied by Lab-Scan (Lisbon, Portugal). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Preparation of Standard Solutions

A stock standard mixture with fructose, glucose, sucrose and raffinose was prepared in water with the final concentration of 100 mg/ml in each compound, and it was used for recovery calculation. Lactose was used as internal standard (IS), being prepared a stock solution at 25 mg/ml in water, kept at −20 °C.

Samples and Sample Preparation

Almond fruits were haphazardly collected in August–September 2008 in orchards located in Trás-os-Montes, Northeast Portugal. Once the available almond trees were located in regions with very similar edaphoclimatic conditions, we opted to collect almonds randomly, and no significant differences were expected among them. Selected plants are not irrigated and no phytosanitary treatments were applied. The chosen cultivars include regional (*Casanova*, *Duro Italiano*, *Pegarinhos one seeded*, *Pegarinhos two seeded* and *Refego*) and commercial (*Ferraduel*, *Ferragnes*, *Ferrastar*, *Gloriette* and *Marcona*) cultivars. The regional almond cultivars belong to *Amêndoa Douro* PDO. The fruits were dried at room temperature and exposed to sun, as common practice in the region.

Regarding chestnuts, five trees were selected in each orchard, and 50 fruits were collected from each tree, according with the tree phonological cycle (chestnuts from *Aveleira* cultivar were collected in October, chestnuts from *Boa Ventura*, *Judia* and *Longal* cultivars were collected in November) during the crop year of 2008. Orchards are located in Trás-os-Montes, in the Northeast of Portugal. The four chestnut cultivars belong to *Castanha da Terra Fria* PDO.

Chestnut and almond fruits were kept at −20 °C and protected from light during about three months. Immediately before the extraction procedure, each sample was manually peeled off (inner and outer skins), incubated at

50 °C until constant weight (≈ 24 h) and then chopped to obtain a fine dried powder (20 mesh).

Extraction Procedures

Crude lipidic fraction was removed from dried and finely chopped chestnuts and almonds (≈50 g in the presence of anhydrous sodium sulfate to retain any residual humidity) extracted with light petroleum ether (bp 40–60 °C) during 16 h in a Universal extraction system B-811 (Büchi, Switzerland); the residual solvent was removed by flushing with nitrogen.

Dried and defatted powder (2.0 g) was spiked with the IS (5 mg/ml), and extracted with 10 ml of 80% aqueous ethanol at 70 °C for 30 min. The resulting suspension was centrifuged at 5,000 rpm for 15 min. The supernatant was concentrated at 40 °C under reduced pressure, until total ethanol removal, and then diluted in water to a final volume of 10 ml.

HPLC Analysis

Free sugars profiles were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI). The HPLC equipment consisted of an integrated system with a Smartline pump 1000, a degasser system Smartline manager 5000, a Smartline 2300 RI detector (2.617×10^{-3} mRIU, 35 °C; Knauer, Germany) and an AS-2057 auto-sampler (Jasco, Japan). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6×250 mm, 5 mm, Knauer) operating at 35 °C (7971R Grace oven). The mobile phase used was acetonitrile/deionized water, 7:3 (*v*/*v*) at a flow rate of 1 ml/min, and the injection volume was 20 µl. The results are expressed in g/100 g of dried weight, calculated by internal standard normalization of the chromatographic peak area. Sugar identification was made by comparing the relative retention times of sample peaks with standards.

Linearity and sensitivity of the HPLC analysis were determined and the method was validated by the instrumental precision, repeatability and accuracy, using *Judia* cultivar for chestnuts and *Ferraduel* cultivar for almonds.

Statistical Analysis

Sugars extraction was performed in duplicate and each sample was injected twice in HPLC-RI. The results are expressed as mean values ± standard deviation. Concerning chestnut fruits, the differences between different cultivars were analyzed using one-way analysis of variance followed by Tukey's honestly significant difference *post hoc* test with $\alpha=0.05$, coupled with Welch's statistic.

Results and Discussions

Table 1 presents the analytical characteristics as well as the method validation parameters of the reported chromatographic method. A 7-level calibration curve was made for each compound using the peak/area ratio between the sugar and lactose versus concentration of the standard (mg/ml). The correlation coefficients were always higher than 0.999 for all the compounds. The limits of detection (LOD), calculated as the concentration corresponding to three times the standard error of the calibration curve divided by the slope, ranged from 0.05 to 0.08 mg/ml. The limits of quantification (LOQ) were calculated using the concentration corresponding to ten times the calibration error divided by the slope, and ranged from 0.18 to 0.25 mg/ml.

The precision of the extraction method was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day precision was evaluated by assaying

Table 1 Analytical characteristics and method validation parameters of the reported method

Parameter		Fructose	Glucose	Sucrose	Lactose (IS)	Raffinose
R_t (retention time)	min	5.97	6.36	7.41	8.87	10.75
	C.V. (%) (n=10)	0.27	0.26	0.33	0.41	0.36
Correlation coefficient (R^2)		0.9999	0.9999	0.9999	–	0.9991
Linearity range (mg/ml)		0.2–24	0.3–24	0.2–24	–	0.3–24
LOD (mg/ml)		0.05	0.08	0.06	–	0.09
LOQ (mg/ml)		0.18	0.25	0.21	–	0.30
Precision C.V. (%) (n=6)		1.42	1.28	0.96	–	1.17
Repeatability C.V. (%) (n=6)		3.23	1.14	3.64	–	2.87
Accuracy C.V. (%) (n=6)		91.90	99.84	89.25	–	88.53

IS Internal standard

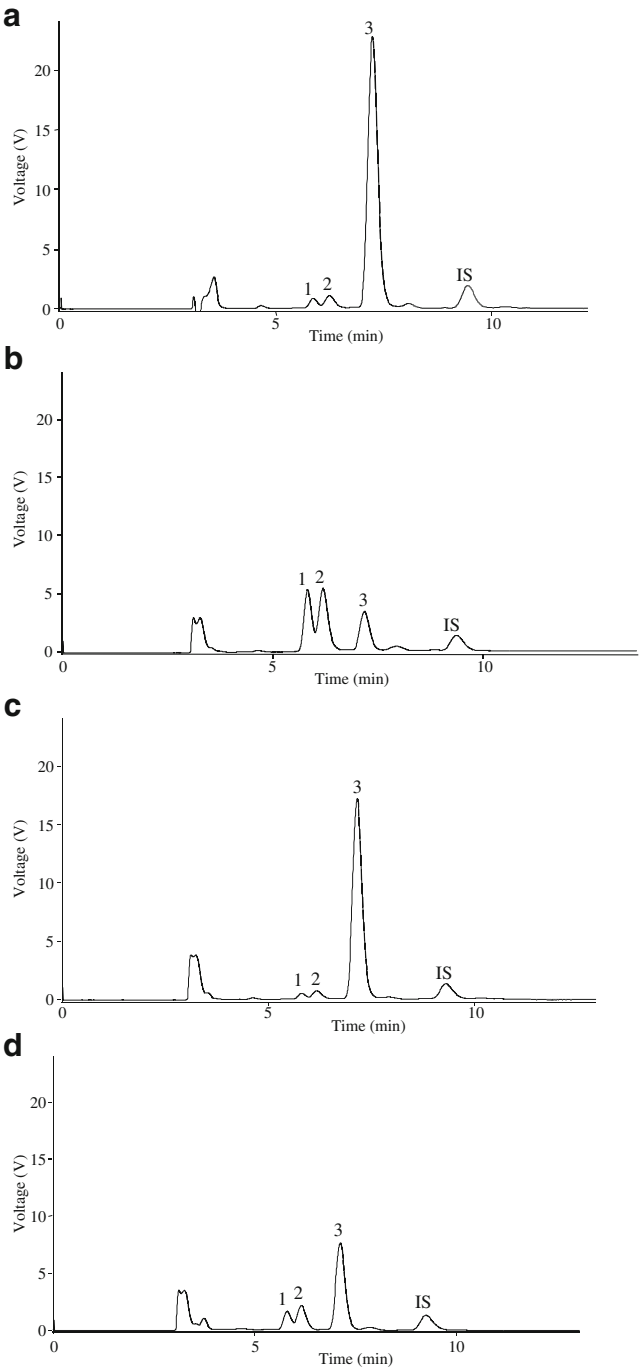

Fig. 1 Chromatograms comparison for assayed chestnut cultivars: **a** *Aveleira*; **b** *Boa Ventura*; **c** *Judia*; **d** *Longal*. *1*- Fructose; *2*- Glucose; *3*- Sucrose; *IS*- Lactose

a sample extracted six times during the same day (coefficients of variation ranged between 0.96 and 1.42%, for all sugars). The inter-day precision was performed by analyzing the same sample in six different and subsequent days (coefficients of variation found varied between 1.14% and 3.64%, for all sugars).

In the absence of a reference matrix, the method accuracy was evaluated by the standard addition procedure (percentage of recovery). The standards mixture was added to the samples in three concentration levels (0.375, 6.0 and 24.0 mg/ml, each one in duplicate) before the extraction. The method showed good recovery values, with mean percentages ranging between 88.53% and 99.84%.

Chestnut cultivars showed a relevant heterogeneity (Fig. 1). Nevertheless, all of them presented fructose, glucose and sucrose. Sucrose was the main sugar in *Aveleira* (22.05±1.48), *Judia* (23.30±0.83) and *Longal* (9.56±0.91), while glucose was prevalent in *Boa Ventura* (6.63±0.49), but in lower extension (Table 2). The prevalence of sucrose is in agreement with the results reported by other authors in Swiss, Italian [10] and Spanish cultivars [11].

In almond cultivars, sucrose was always the main sugar (11.46±0.14 in *Marcona* to 22.23±0.59 in *Ferragnes* g/100 g of dried weight) (Table 3) and the sugars profiles

Table 2 Sugar composition (g/100 g of dry weight) of chestnut cultivars (mean ± standard deviation). In each column, and in the mean values of each cultivar, different letters mean significant differences ($p<0.05$)

Cultivar		Fructose	Glucose	Sucrose
Aveleira	1	0.72±0.05	1.14±0.06	24.17±0.17
	2	0.64±0.00	1.03±0.00	23.24±0.15
	3	0.70±0.02	1.08±0.02	20.88±0.13
	4	0.68±0.01	0.99±0.00	20.91±0.28
	5	0.84±0.03	1.25±0.01	21.05±0.46
	x	0.72±0.07 c	1.10±0.10 c	22.05±1.48 b
Boa Ventura	1	5.11±0.06	6.38±0.09	4.07±0.22
	2	5.32±0.09	6.79±0.03	3.71±0.06
	3	5.28±0.10	6.81±0.07	3.87±0.05
	4	4.84±0.06	6.21±0.09	4.02±0.04
	5	4.88±0.05	6.24±0.07	4.21±0.11
	x	5.18±0.39 a	6.63±0.49 a	4.03±0.30 d
Judia	1	0.63±0.07	1.05±0.09	22.96±1.80
	2	0.68±0.03	1.09±0.02	23.67±0.10
	3	0.57±0.01	0.96±0.00	23.44±1.06
	4	0.59±0.02	0.97±0.02	22.68±0.29
	5	0.62±0.00	1.03±0.01	23.76±0.08
	x	0.62±0.05 c	1.02±0.06 c	23.30±0.83 a
Longal	1	1.76±0.16	2.84±0.26	9.50±1.30
	2	1.76±0.00	2.56±0.00	8.67±0.03
	3	1.92±0.15	2.96±0.22	9.20±0.83
	4	1.92±0.03	2.66±0.06	10.78±0.38
	5	1.69±0.02	2.45±0.05	9.64±0.20
	x	1.81±0.12 b	2.69±0.23 b	9.56±0.91 c

Table 3 Sugar composition (g/100 g of dry weight) of almond cultivars (mean ± standard deviation)

Cultivar	Fructose	Glucose	Sucrose	Raffinose
Casanova	0.24±0.03	0.96±0.14	13.93±0.73	1.93±0.17
Duro Italiano	0.27±0.02	1.11±0.12	13.18±0.92	2.11±0.29
Ferraduel	0.38±0.03	0.95±0.02	16.25±0.76	0.71±0.05
Ferragnes	0.37±0.03	1.47±0.19	22.23±0.59	0.75±0.12
Ferrastar	0.17±0.02	1.04±0.08	21.97±1.39	1.73±0.19
Gloriette	0.59±0.05	1.30±0.32	16.87±0.45	0.89±0.08
Marcona	0.29±0.03	0.77±0.04	11.46±0.14	1.67±0.13
Pegarinhos one seeded	0.19±0.05	0.71±0.13	11.99±1.46	1.43±0.21
Pegarinhos two seeded	0.11±0.02	0.42±0.12	15.87±0.83	1.29±0.19
Refego	0.25±0.03	0.68±0.14	14.73±1.13	1.40±0.10

were much more homogeneous (Fig. 2). The prevalence of sucrose as the main sugar in almond is in agreement with previous works [2, 12]. The commercial cultivars proved to have higher sucrose contents, only with the exception of *Marcona*. Raffinose was the second major sugar (0.71± 0.05 in *Ferraduel* to 2.11±0.29 in *Duro Italiano*), followed by glucose (0.42±0.12 in *Pegarinhos two seeded* to 1.47± 0.19 in *Ferragnes*) and fructose (0.11±0.02 in *Pegarinhos two seeded* to 0.59±0.05 in *Gloriette*). Generally, more than 95% of sugars were identified for both matrixes. These results are in conformity with previous studies, in Spanish cultivars where sucrose and raffinose were found in high amounts in almond kernels, while reducing sugars (fructose and glucose) and sugar alcohols (inositol and sorbitol) were found only in trace amounts [13]. Actually, soluble sugars are stored as sucrose and raffinose in fully developed kernels [14].

The differences among glucose and fructose contents indicate that these reducing sugars should exist naturally in almond, once that if they were formed exclusively after sucrose hydrolysis, they should be present in similar amounts.

Overall, some studies have already been applied to sugar composition in chestnut, however, and as far as we know, this is the first study about *Aveleira*, *Boa Ventura* and *Judia* sugars composition; even so, the results obtained for *Longal* are in agreement with other reported work [10] with this cultivar, obtained in a different geographic location. These results are potentially useful from the commercial point of view, because sugars profiles might be useful on inter-cultivar discrimination, enhancing the possibilities of acquiring a valuable authenticity factor.

Regarding almond, commercial and regional cultivars seems to have different sugar composition, especially in sucrose amounts. However, the obtained profiles revealed a much higher homogeneity in quantitative terms. Besides characterization purposes, sugars profiles, and especially sucrose, may also be used as an indicator of chestnut and almond quality, once that this disaccharide is one of the most important parameters in the assessment of adequate storage conditions and fruit quality.

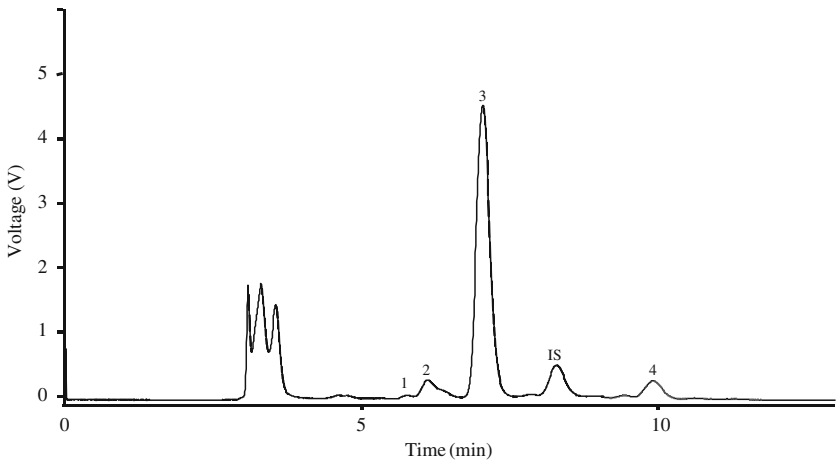

Fig. 2 Almond cultivar (*Ferrastar*) chromatogram example. *1*- Fructose; *2*- Glucose; *3*- Sucrose; *4*- Raffinose; *IS*- Lactose

Free sugars are important nutritional components that affect the kernel flavor of almond. The nutritional improvement of nut crops through breeding efforts will gain increasing importance in promoting a more healthful lifestyle [15].

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