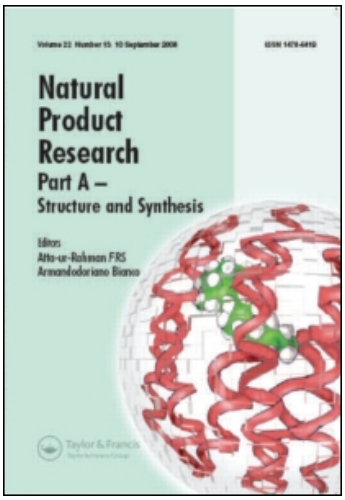

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Relevant principal component analysis applied to the characterisation of Portuguese heather honey

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Relevant principal component analysis applied to the characterisation of Portuguese heather honey

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The main purpose of this study was the characterisation of 'Serra da Lousã' heather honey by using novel statistical methodology, relevant principal component analysis, in order to assess the correlations between production year, locality and composition. Herein, we also report its chemical composition in terms of sugars, glycerol and ethanol, and physicochemical parameters. Sugars profiles from 'Serra da Lousã' heather and 'Terra Quente de Trás-os-Montes' lavender honeys were compared and allowed the discrimination: 'Serra da Lousã' honeys do not contain sucrose, generally exhibit lower contents of turanose, trehalose and maltose and higher contents of fructose and glucose. Different localities from 'Serra da Lousã' provided groups of samples with high and low glycerol contents. Glycerol and ethanol contents were revealed to be independent of the sugars profiles. These data and statistical models can be very useful in the comparison and detection of adulterations during the quality control analysis of 'Serra da Lousã' honey.

Keywords: 'Serra da Lousã' heather honey; relevant principal component analysis; sugars profile; glycerol content; ethanol content

1. Introduction

Nowadays, consumers' preferences are for food products that are very specific and this restricts quality criteria. The floral origin of honey is one of the main factors that define its quality, being closely related with the geographical provenience and season of collection.

In general, monofloral honeys are more expensive than multifloral ones (Andrade et al., 1999; Ferreres, Andrade, Gil, & Tomas-Barberan, 1996b). Some monofloral honeys are appreciated more than others due to their organoleptic properties or their pharmacological attributes. In Portugal, consumer choice is for heather honey, which is

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considered to be superior to other types that are locally produced or imported from other countries. Heather honey is produced in Portugal from *Erica* sp. (Ericaceae), while, for example, in Spain or France, it comes from either *Calluna* or *Erica* sp. (Andrade et al., 1999).

The standard procedure for assessing a honey's botanical origin is melissopalynology, which consists of the microscopical analysis of pollen present in the honey after filtration or centrifugation (Cometto, Faye, Di Paola Naranjo, Rubio, & Aldao, 2003). To achieve reliable results, this technique requires a specialised technician with previous knowledge of pollen morphology. Besides, melissopalynology is difficult to apply in filtered processed honey because of pollen scarcity or lack of pollen (Baroni, Chiabrando, Costa, & Wunderlin, 2002). Therefore, the search for alternative methodology, which allows the establishment of correlations between the honey's floral origin and the presence of certain compounds of nectar origin or resulting from the biochemical modification of nectar compounds by the bees, is of great practical importance to evaluate honey quality.

In the past few years there has been an increasing interest in finding objective analytical methods that could complement or even substitute melissopalynology on the determination of a honey's floral origin (Andrade, Ferreres, & Amaral, 1997; Andrade, Ferreres, Gil, & Tomas-Barberan, 1997; Andrade et al., 1999; Baroni et al., 2002; Cometto et al., 2003; Cotte, Casabianca, Chardon, Lheritier, & Grenier-Loustalot, 2004a; Cotte et al., 2004b; Ferreres, Andrade, & Tomas-Barberan, 1994; Ferreres et al., 1996b; Nozal et al., 2005; Rashed & Soltan, 2004; Ruoff et al., 2005; Tewari & Irudayaraj, 2005). The evaluation of the quality, origin and authenticity of honey usually involves the measurement of standard physical and chemical parameters, such as pH, acidity, moisture, hydroxymethylfurfural (HMF), diastases activity and sugars profile (Baroni et al., 2002; Cometto et al., 2003; Nozal et al., 2005). Other less-frequently analysed parameters are amino acids, proteins, flavonoids and phenolic acids (Baroni et al., 2002). Primary normal alcohols (Huidobro et al., 1994) and glycerol (Huidobro et al., 1993) are minor constituents that have also been determined to evaluate honey quality, once their amounts rise with aging. The use of multiparametric studies, associated with chemometrics, yields satisfactory results for honey classification (Andrade et al., 1999; Baroni et al., 2002; Cometto et al., 2003; Cotte et al., 2004a; Cotte et al., 2004b; Nozal et al., 2005; Rashed & Soltan, 2004; Ruoff et al., 2005; Tewari & Irudayaraj, 2005).

The 'Serra da Lousã' region (central Portugal) is known for the high quality of its heather honey, which gained the *Denomination d'Origine Controllée* (DOC). Heather honey produced in this region has been extensively studied by our research group in order to examine its pollen spectrum, several physicochemical parameters (Andrade et al., 1999), flavonoids (Ferreres et al., 1994; Ferreres et al., 1996b), phenolic acids (Andrade et al., 1997; Ferreres et al., 1996b) and abscisic acid (Ferreres, Andrade, & Tomas-Barberan, 1996a).

This article herein reports, for the first time, the primary normal alcohols and glycerol contents and the sugars profile of 'Serra da Lousã' heather honey samples, collected in the same beehives of 20 distinct localities of this region during three consecutive years. In addition, its sugars profile was compared with that of 'Terra Quente de Trás-os-Montes' lavender honey, another honey that is very popular in Portugal.

As far as we know, there is no other study with such a high number of samples from exactly the same origin or collected for such a long period. So, the use of relevant principal components analysis (RPCA) for these data can be advantageous and provide valuable information about the characteristics of 'Serra da Lousã' heather honey, allowing us to evaluate the influence of the production year and the local provenience in its qualitative and quantitative composition, in terms of primary normal alcohols and glycerol contents and sugars profile (data block 1), and in its physicochemical parameters determined before (data block 2) (Andrade et al., 1999). All this information can be useful for both regulatory entities and consumers, as it could serve as database for the detection of adulteration in 'Serra da Lousã' honey.

2. Results and discussion

2.1. *Block 1, sugars profile and ethanol and glycerol contents*

2.1.1. *Sugars profile*

The HPLC analysis of *Erica* sp. honey samples, collected between 1991 and 1993 in the 'Serra da Lousã' region, allowed the detection of fructose, glucose, turanose, maltose, trehalose, isomaltose, raffinose and melezitose.

Quantification of the compounds showed that fructose was the major compound, followed by glucose (Tables 1–3). The absence of sucrose in these *Erica* sp. honeys confirms their nectar source (Andrade, 1995). The oligosaccharides present in these honeys result from the enzymatic activity of the α- and β-glycosidase added to the nectar by the bees (Low, Nelson, & Sporns, 1998). The small amounts of melezitose found in some samples can be attributed to the use of honeydew during honey production.

As mentioned before, fructose and glucose are the major sugars of 'Serra da Lousã' honey (Tables 1–3). All samples contained more fructose than glucose, indicating that *Erica* sp. honeys from the 'Serra da Lousã' region would be less prone to granulation (Ojeda de Rodriguez, Sulbaran de Ferrer, & Rodriguez, 2004). Fructose and glucose contents maintained a relatively constant level from 1991 to 1993, for all the localities (Tables 1–3).

The maltose content was reduced between 1991 and 1993, from 3.16 ± 1.18 to 1.19 ± 0.22 ($p < 10^{-6}$). A similar decrease was found for isomaltose ($p < 0.10$, Tables 1–3). These results are not surprising considering that both sugars are glucose disaccharides.

The following sugars presented higher Pearson correlation coefficients: (i) maltose and isomaltose (0.7903); (ii) raffinose and trehalose (0.8715); and (iii) fructose/glucose ratio and total sugars content (0.8715).

2.1.2. *Comparison between 'Serra da Lousã' heather and 'Terra Quente de Trás-os-Montes' lavender honeys sugars profiles*

Heather honey from 'Serra da Lousã' is characterised by the absence of both sucrose and melibiose. Therefore, the presence of these sugars in 'Serra da Lousã' honey are considered an adulteration. In *Lavender stoechas* L. honeys produced at 'Terra Quente de Trás-os-Montes' in 1991 (Table 4), the presence of both sugars was found.

Table 1. Sugar composition of 'Serra da Lousã' honey samples from 1991 (g/100 g of honey).

Sample No.	Fructose	Glucose	Sucrose	Turanose	Maltose	Trehalose	Isomaltose	Raffinose	Melibiose	Melezitose
1	42.40	26.40	–	0.02	3.84	0.03	2.60	–	–	0.18
2	43.42	31.50	–	0.23	5.75	0.14	7.40	2.61	–	0.18
3	42.00	25.50	–	0.04	3.72	0.10	4.54	–	–	–
4	47.00	27.60	–	0.04	2.01	0.04	3.66	–	–	1.20
5	39.90	23.30	–	0.48	5.28	0.01	4.20	–	–	1.00
6	42.60	29.90	–	0.03	2.58	0.03	2.62	–	–	–
7	33.40	21.00	–	0.001	3.39	0.03	2.64	–	–	–
8	42.90	31.20	–	0.04	0.93	0.03	1.46	–	–	–
9	44.10	23.70	–	0.06	3.36	0.03	3.72	–	–	–
10	44.10	31.80	–	0.11	3.24	0.09	2.76	–	–	–
11	40.70	27.00	–	0.07	4.11	0.06	4.34	–	–	–
12	38.80	25.50	–	0.07	3.48	0.06	2.10	–	–	0.47
13	41.00	27.00	–	0.02	4.23	0.03	2.48	1.44	–	0.48
14	48.00	31.20	–	0.02	3.54	0.03	2.80	–	–	–
15	42.00	22.50	–	0.06	2.85	0.04	2.62	–	–	–
16	48.60	34.50	–	0.06	3.00	0.03	2.40	–	–	–
17	48.30	33.00	–	0.04	2.19	0.03	2.60	–	–	–
18	44.10	30.90	–	0.05	1.74	0.02	1.36	–	–	–
19	48.30	36.00	–	0.03	2.04	0.004	1.88	–	–	–
20	37.20	30.00	–	0.02	2.01	0.02	2.40	–	–	–
$\overline{X}$[a]	42.94	28.48	–	0.07	3.16	0.04	3.03	2.03	–	0.59
SD[b]	3.95	4.15	–	0.11	1.18	0.03	1.35	0.83	–	0.42
Minimum	33.40	21.00	–	0.001	0.93	0.004	1.36	1.44	–	0.18
Maximum	48.60	36.00	–	0.48	5.75	0.14	7.40	2.61	–	1.20

Notes: [a]$\overline{X}$: mean.
[b]SD: standard deviation.

Table 2. Sugar composition of 'Serra da Lousã' honey samples from 1992 (g/100 g of honey).

Sample No.	Fructose	Glucose	Sucrose	Turanose	Maltose	Trehalose	Isomaltose	Raffinose	Melibiose	Melezitose
1	43.68	34.71	–	0.04	3.61	0.03	4.22	–	–	0.11
2	46.70	34.58	–	0.07	5.23	0.03	4.72	1.32	–	0.16
3	52.65	34.52	–	0.10	4.37	0.03	5.85	–	–	–
4	41.81	29.41	–	0.06	3.58	0.003	4.94	–	–	–
5	42.52	26.67	–	0.07	2.99	0.01	4.86	–	–	0.93
6	48.39	33.68	–	0.08	3.93	0.05	5.64	–	–	–
7	47.80	31.43	–	0.10	5.65	0.01	6.08	–	–	–
8	44.00	29.40	–	0.07	5.42	0.01	5.35	–	–	–
9	37.21	27.24	–	0.01	2.83	0.02	2.73	–	–	–
10	43.70	29.23	–	0.07	2.11	0.07	3.56	–	–	–
11	47.17	37.81	–	0.04	2.05	0.06	3.04	–	–	–
12	48.51	34.21	–	0.15	4.47	0.05	5.34	–	–	0.27
13	46.76	32.90	–	0.08	3.96	0.03	5.31	1.02	–	0.20
14	46.41	38.42	–	0.23	3.66	0.18	3.88	–	–	–
15	44.45	31.09	–	0.06	3.54	0.03	4.58	–	–	–
16	43.01	35.33	–	0.00	1.39	0.04	1.69	–	–	–
17	50.10	34.87	–	0.08	4.06	0.05	5.84	–	–	–
18	42.59	27.37	–	0.04	1.69	0.03	2.65	–	–	–
19	38.04	30.64	–	0.00	1.03	0.02	2.62	0.93	–	–
20	45.81	34.70	–	0.02	1.44	0.04	1.43	–	–	–
$\overline{X}$[a]	45.07	32.41	–	0.07	3.35	0.04	4.22	0.16	–	0.08
SD[b]	3.77	3.43	–	0.05	1.38	0.04	1.43	0.41	–	0.21
Minimum	37.21	26.67	–	0.004	1.03	0.003	1.43	0.93	–	0.11
Maximum	52.65	38.42	–	0.23	5.65	0.18	6.08	1.32	–	0.93

Notes: [a]$\overline{X}$: mean.
[b]SD: standard deviation.

Table 3. Sugar composition of 'Serra da Lousã' honey samples from 1993 (g/100 g of honey).

Sample No.	Fructose	Glucose	Sucrose	Turanose	Maltose	Trehalose	Isomaltose	Raffinose	Melibiose	Melezitose
1	48.73	37.94	–	0.02	1.53	0.12	2.77	–	–	0.17
2	41.97	29.85	–	0.04	1.12	0.03	2.75	2.39	–	0.20
3	48.98	39.70	–	0.01	1.19	0.09	3.15	–	–	0.98
4	48.10	30.31	–	0.09	1.51	0.07	2.51	–	–	1.09
5	48.22	37.43	–	0.02	1.46	0.10	3.02	–	–	–
6	42.14	32.51	–	0.01	1.16	0.003	1.31	–	–	–
7	36.37	28.02	–	0.00	1.04	0.05	3.56	–	–	–
8	49.23	39.43	–	0.02	0.88	0.05	2.69	–	–	–
9	41.61	31.44	–	0.03	1.27	0.03	3.04	–	–	–
10	45.37	36.12	–	0.04	1.43	0.07	2.70	–	–	–
11	45.75	23.21	–	0.04	1.41	0.06	1.27	–	–	–
12	41.14	31.85	–	0.001	1.00	0.04	1.40	–	–	0.25
13	40.44	29.15	–	0.07	1.08	0.05	2.24	1.88	–	0.51
14	45.27	33.13	–	0.02	0.81	0.03	1.45	–	–	–
15	46.81	28.31	–	0.04	1.24	0.03	2.02	–	–	–
16	48.88	37.75	–	0.07	1.08	0.05	2.25	–	–	–
17	47.09	32.14	–	0.05	0.91	0.04	0.92	–	–	–
18	48.68	32.62	–	0.04	1.32	0.13	1.11	–	–	–
19	39.67	28.69	–	0.06	1.35	0.05	2.11	–	–	–
20	44.87	33.38	–	0.02	0.95	0.05	1.65	–	–	–
$\overline{X}$[a]	44.97	32.65	–	0.03	1.19	0.06	2.20	0.21	–	0.16
SD[b]	3.78	4.34	–	0.02	0.22	0.03	0.77	0.66	–	0.33
Minimum	36.37	23.21	–	0.001	0.81	0.003	0.92	1.88	–	0.17
Maximum	49.23	39.70	–	0.09	1.53	0.13	3.56	2.39	–	1.09

Notes: [a]$\overline{X}$: mean.
[b]SD: standard deviation.

Table 4. Sugar composition of 'Terra Quente of Trás-os-Montes' honey samples from 1991 (g/100 g of honey).

Sample No.	Fructose	Glucose	Sucrose	Turanose	Maltose	Trehalose	Isomaltose	Raffinose	Melibiose	Melezitose
1	39.60	28.50	2.04	0.33	4.53	0.52	0.72	–	0.11	–
2	35.02	23.40	1.44	0.48	2.88	0.34	0.34	–	0.10	–
3	37.20	21.30	0.72	0.66	2.04	0.20	0.48	–	0.11	–
4	41.14	30.00	0.84	0.69	7.83	0.80	0.60	–	0.28	–
5	35.15	28.20	3.00	0.30	3.90	0.20	0.17	–	0.11	–
6	34.04	27.60	3.00	0.26	3.39	0.08	0.16	–	0.16	–
7	43.29	31.20	1.68	0.33	4.98	0.46	2.04	–	0.05	–
8	44.40	30.90	0.54	0.48	6.06	0.52	0.18	–	0.03	–
9	38.11	30.60	0.01	0.15	1.20	0.20	0.16	–	0.03	–
10	48.80	30.30	0.60	0.45	5.91	0.52	0.22	–	0.10	–
11	34.00	30.00	1.80	0.39	4.22	0.22	0.20	–	0.08	–
12	45.50	31.20	0.50	0.42	5.16	0.62	0.22	–	0.03	–
13	43.20	27.00	0.59	0.33	4.23	0.24	0.30	–	0.03	–
14	40.40	27.90	0.36	0.33	4.26	0.30	0.10	–	0.03	–
15	46.80	30.00	0.30	0.18	1.71	0.22	0.12	–	0.02	–
16	48.80	31.20	0.30	0.30	1.92	0.22	0.14	–	0.03	–
17	43.20	27.90	0.31	0.27	2.73	0.24	0.13	–	0.03	–
18	35.20	26.40	2.64	0.30	4.14	0.20	0.12	–	0.12	–
19	48.10	33.30	0.31	0.28	2.90	0.14	0.15	–	0.03	–
20	37.06	27.60	0.27	0.36	2.37	0.28	0.17	–	0.03	–
$\overline{X}$[a]	40.95	28.73	1.06	0.36	3.82	0.33	0.34	–	0.08	–
SD[b]	5.10	2.82	0.97	0.14	1.67	0.19	0.43	–	0.06	–
Minimum	34.00	21.30	0.01	0.15	1.20	0.08	0.10	–	0.02	–
Maximum	48.80	33.30	3.00	0.69	7.83	0.80	2.04	–	0.28	–

Notes: [a]$\overline{X}$: mean.
[b]SD: standard deviation.

Figure 1 presents the principal components (PCs) Gabriel plot and the loadings hierarchical clustering for the honey sugars profiles. The most significant difference between the two types of honey is in sucrose content. 'Serra da Lousã' honeys do not present this sugar in their composition, while those from 'Terra Quente de Trás-os-Montes' do. Therefore, RPCA was performed by taking into account the rest of the sugars profile. The RPCA algorithm has obtained three PCs, accounting for 89.27% of total variance at 0.99% of significance level. PC1 (46.96%) discriminates the effects on the variance of turanose, trehalose and maltose against isomaltose, glucose and fructose. PC2 (23.45%) presents the differences between high and low sugar content, and PC3 (18.86%) discriminates the importance of maltose and isomaltose against the rest of the sugars profile.

Generally, 'Terra Quente de Trás-os-Montes' honeys present higher contents of turanose, trehalose and maltose when compared with those from 'Serra da Lousã' ($p < 0.001$). Furthermore, 'Serra da Lousã' honey tends to contain higher contents of fructose and glucose ($p < 0.001$), as shown in the Gabriel plot (Figure 1a). No significant differences were observed in the 'Serra da Lousã' sugars composition from 1991 to 1993. Such result reveals that sugars profile (Tables 1–3) can be used for the discovery of adulterations and quality control.

The Gabriel plot of PC2 *versus* PC3 (Figure 1b) shows how the variance in the sugars profile of both honeys is structured. No significant differences were obtained on the scores of both 'Terra Quente de Trás-os-Montes' and 'Serra da Lousã' honeys, as well as between 1991 and 1993.

The two honeys preserve the same type of relationships between the different sugars (Figure 1b). Such a structure is observable in the hierarchical clustering of Figure 1(c), where it is possible to derive the following relationships: (i) fructose and glucose, (ii) turanose and trehalose and (iii) maltose and isomaltose. Such relationships can be used to detect abnormal changes in 'Serra da Lousã' and 'Terra Quente de Trás-os-Montes' honeys, and can be important information for quality control.

2.1.3. *Primary normal alcohols contents*

Ethanol is naturally present in honey in small quantities. It occurs due to the fermentation process, together with carbon dioxide and several volatile and non-volatile acids (Marvin, 1958). Primary normal alcohols (ethanol, 1-propanol, butanol, pentanol and other primary alcohols) have been determined as apparent ethanol contents, which range from a minimum of 3.92 to a maximum of 27.8 mg kg^{-1} (Tables 5–7). These values are lower than those found for Galician (Spanish) (Huidobro et al., 1994) and French (Cotte et al., 2004a) honey samples. No significant differences were observed between samples from 1991 and 1993. However, honeys obtained in two different localities presented abnormal ethanol amounts ($p < 0.01$): Arganil-Coja, with high ethanol content (22.12 ± 5.67 mg kg^{-1}), and Vila Nova de Poiares, with low ethanol content (4.18 ± 0.35 mg kg^{-1}).

2.1.4. *Glycerol contents*

Glycerol is a minor honey constituent, thought to be produced by nectar and honeydew microorganisms. Yeast is capable of fermenting honey glucose to glycerol to a considerable extent (Spencer & Sallans, 1956). The honey samples presented glycerol

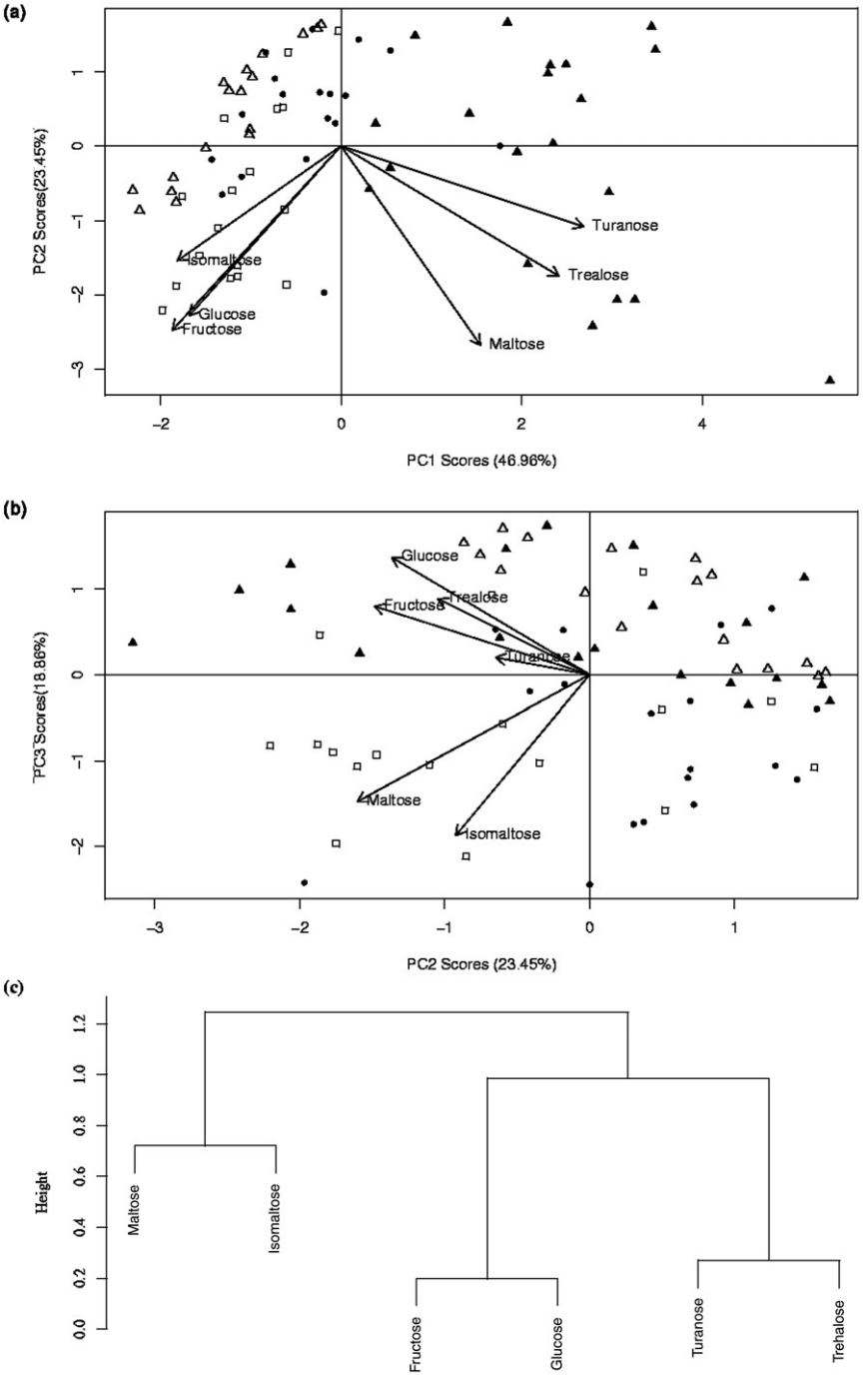

Figure 1. Comparison between 'Serra da Lousã' and 'Terra Quente of Trás-os-Montes' honey sugars profiles. Gabriel plot of (a) PC1 (46.96%) *vs*. PC2 (23.45%) and (b) PC3 (18.86%) *vs*. PC2 (23.45%). For 'Serra da Lousã': ● 1991, □ 1992 and Δ 1993. For 'Terra Quente de Trás-os-Montes': ▲1991. (c) The first five PCs loading hierarchical clustering dendogram.

Table 5. Ethanol content of 'Serra da Lousã' honey samples from 1991.

Sample	Ethanol mg kg^{-1} honey	Sample	Ethanol mg kg^{-1} honey
1	7.77	11	10.97
2	8.29	12	12.60
3	3.92	13	9.90
4	13.73	14	9.84
5	11.52	15	10.19
6	7.12	16	12.50
7	6.98	17	13.01
8	27.80	18	13.57
9	11.49	19	8.83
10	11.46	20	6.75
		Mean:	10.91
		SD:	4.76
		Minimum:	3.92
		Maximum:	27.80

Table 6. Ethanol content of 'Serra da Lousã' honey samples from 1992.

Sample	Ethanol mg kg^{-1} honey	Sample	Ethanol mg kg^{-1} honey
1	8.34	11	9.84
2	4.59	12	12.85
3	11.63	13	11.05
4	11.63	14	8.32
5	12.03	15	10.65
6	7.88	16	13.53
7	9.06	17	11.96
8	16.63	18	10.01
9	10.33	19	7.83
10	12.16	20	8.32
		Mean:	10.32
		SD:	2.59
		Minimum:	4.59
		Maximum:	16.63

content between 34.09 and 148.65 mg kg^{-1} (Tables 8–10). These results are within the values obtained by other authors (Huidobro et al., 1993), being a highly variable parameter.

Significant differences were found between the samples obtained in different localities of 'Serra da Lousã', making it possible to discriminate them into two different groups ($p < 0.001$): samples with (i) high glycerol content [Góis (113.21 ± 4.09 mg kg^{-1}), Penela (78.98 ± 7.28 mg kg^{-1}), Pampilhosa da Serra (131.22 ± 16.56 mg kg^{-1}), Castanheira de Pêra (93.98 ± 3.87 mg kg^{-1}), Arganil-Rochel (95.45 ± 6.82 mg kg^{-1}), Arganil-Pombeiro da

Table 7. Ethanol content of 'Serra da Lousã' honey samples from 1993.

Sample	Ethanol mg kg^{-1} honey	Sample	Ethanol mg kg^{-1} honey
1	6.93	11	11.04
2	8.45	12	11.99
3	4.03	13	9.09
4	13.78	14	10.06
5	10.31	15	9.12
6	6.91	16	13.01
7	8.57	17	12.85
8	23.98	18	11.51
9	11.57	19	8.01
10	11.21	20	7.11
		Mean:	10.48
		SD:	4.02
		Minimum:	4.03
		Maximum:	23.98

Table 8. Glycerol content of 'Serra da Lousã' honey samples from 1991.

Sample	Glycerol mg kg^{-1} honey	Sample	Glycerol mg kg^{-1} honey
1	51.74	11	113.15
2	112.88	12	114.68
3	34.09	13	38.87
4	84.90	14	48.12
5	115.69	15	53.44
6	93.12	16	44.44
7	67.90	17	48.42
8	45.81	18	82.90
9	93.58	19	89.60
10	47.16	20	54.30
		Mean:	71.74
		SD:	28.48
		Minimum:	34.09
		Maximum:	115.69

Beira (113.38 ± 7.52 mg kg^{-1}), Pedrogão Grande-Louriceira (121.73 ± 13.99 mg kg^{-1}), Lousã-Padrão (84.89 ± 7.63 mg kg^{-1}) and Lousã-Foz de Arouce (94.42 ± 5.56 mg kg^{-1})], and samples with (ii) low glycerol content [Miranda do Corvo (54.09 ± 5.54 mg kg^{-1}), Vila Nova de Poiares (37.87 ± 3.54 mg kg^{-1}), Arganil-Piodão (72.43 ± 5.72 mgkg^{-1}), Arganil-Coja (46.47 ± 6.43 mg kg^{-1}), Arganil-Mourão (53.58 ± 7.11 mg kg^{-1}), Pedrogão Grande-Romão (41.08 ± 2.13 mg kg^{-1}), Figueiró dos Vinhos-Campelos (56.77 ± 9.77 mg kg^{-1}), Figueiró dos Vinhos-Arraçais (53.22 ± 2.21 mg kg^{-1}), Lousã-Favariça (49.58 ± 4.94 mg kg^{-1}), Lousã-Cerdeira (54.14 ± 8.65 mg kg^{-1}) and Lousã-Sarnadinhos (56.49 ± 3.91 mg kg^{-1})].

Table 9. Glycerol content of 'Serra da Lousã' honey samples from 1992.

Sample	Glycerol mg kg^{-1} honey	Sample	Glycerol mg kg^{-1} honey
1	60.43	11	121.01
2	117.45	12	137.85
3	41.10	13	43.12
4	70.85	14	67.36
5	148.65	15	59.22
6	97.38	16	50.00
7	78.85	17	64.10
8	53.21	18	93.33
9	103.02	19	100.51
10	61.23	20	71.37
		Mean:	82.00
		SD:	31.39
		Minimum:	41.10
		Maximum:	148.65

Table 10. Glycerol content of 'Serra da Lousã' honey samples from 1993.

Sample	Glycerol mg kg^{-1} honey	Sample	Glycerol mg kg^{-1} honey
1	50.12	11	105.97
2	109.30	12	112.67
3	38.42	13	41.25
4	81.20	14	54.83
5	129.33	15	47.01
6	89.65	16	54.30
7	70.53	17	49.90
8	40.39	18	78.46
9	89.77	19	93.17
10	52.36	20	43.81
		Mean:	71.62
		SD:	28.23
		Minimum:	38.42
		Maximum:	129.33

Samples from the same geographic origin exhibited the highest glycerol amount in 1992 (Table 9), which could be due to the presence of a raised content of *Eucalyptus globulus* L. pollen in those honeys, related to the use of its nectar by the bees (Andarde, 1995). Such observation is plausible, because there was an abnormal scarcity of *Erica* sp. and the honey bees searched another nectar source, especially *E. globulus* L.

An RPCA including the sugars profile, ethanol and glycerol, was performed to assess a better understanding of the relationship between these parameters. The RPCA algorithm

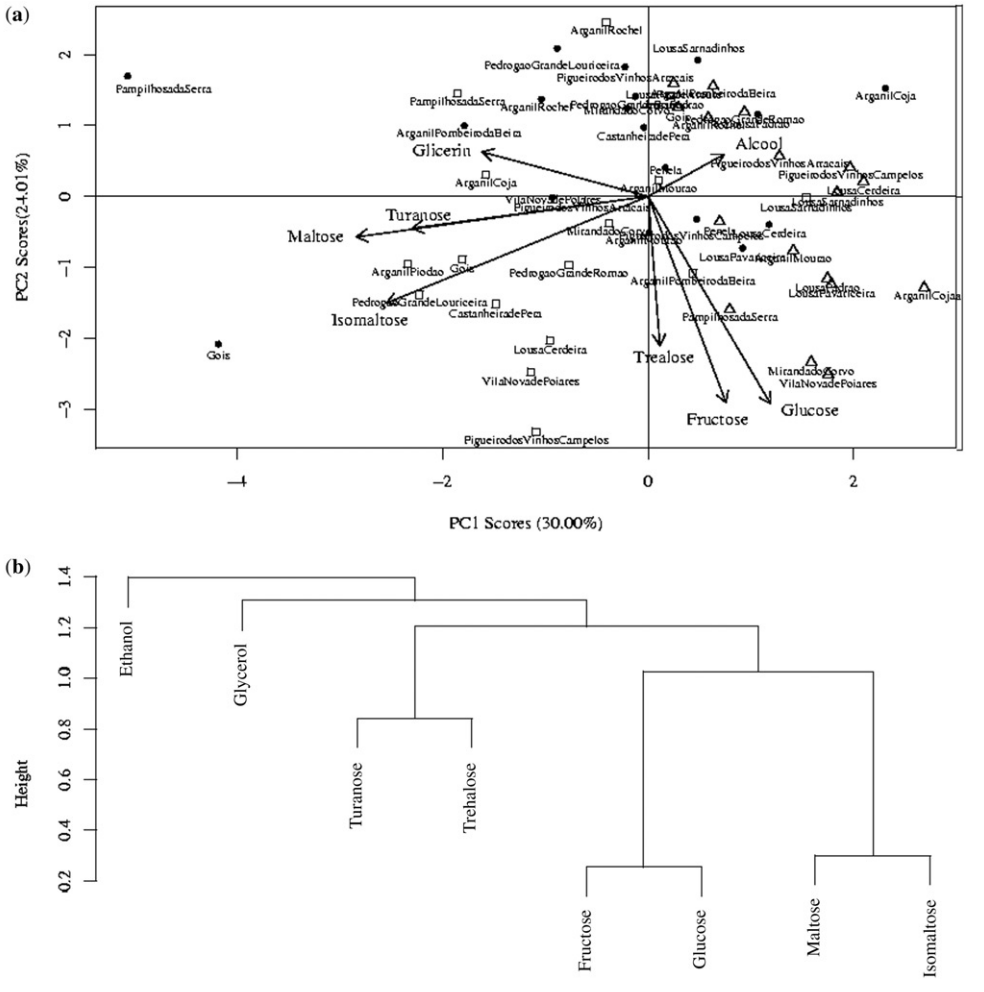

Figure 2. 'Serra da Lousã' honey sugars profile, and ethanol and glycerol contents PCA: (a) Gabriel plot of PC1 (30.00%) *vs.* PC2 (24.01%), with: ● 1991, □ 1992 and Δ 1993. (b) The first five PCs loadings hierarchical clustering dendogram.

obtained five RPCs, which explain 88.88% of total variance. Figure 2(a) presents the first two RPCs, which account for 54.01% of total variance. The year 1993 exhibited the lowest content of maltose ($p < 0.001$), and the most important RPC1 loadings are given by maltose, isomaltose and turanose. Although the latest two do not allow honey samples discrimination in terms of year or locality, they express an important part of total variance. In RPC2 it can be observed that the year 1991 presents significantly lower quantities of glucose ($p < 0.001$) and fructose ($p < 0.01$). Trehalose presents significant loadings on PC2 to PC6, and it is also an important source of variation.

Glycerol discrimination between the different years and localities is present both in RPC1, RPC2, RPC4 (12.23%) and RPC5 (9.15%). Some geographic discrimination is

present in the Gabriel plot of RPC1 *versus* RPC2 (Figure 2a) (54.01% of total variance), while the effect of the production year is more observable on the Gabriel plot of RPC4 *versus* RPC5 (21.38% of total variance). This observation leads to the conclusion that geographical location presents a higher contribution to the honey's chemical variance than the production year.

Figure 2(b) presents the first five RPCs loading hierarchical clustering dendograms of Block 1. It is possible to observe that sugars form the following groups: (i) maltose and isomaltose, (ii) fructose and glucose and (iii) turanose and trehalose. Furthermore, maltose and isomaltose are orthogonal to all the other sugar loadings, meaning that their concentration is independent of the rest of the sugars profile.

Glycerol and ethanol loadings are well distanced from the analysed sugars, which seems to indicate that their content is independent of the sugars profile in 'Serra da Lousã' honey. This result was theoretically not expected, but is statistically significant, being an observable characteristic of 'Serra da Lousã' honey.

2.2. *Block 2, physicochemical parameters*

According to a previous work (Andrade, 1999) the honey quality, evaluated by the determination of pollen spectrum and physicochemical attributes, was found to meet all major national (NP 1307 and 1309, 1976) and international honey specifications (Boletin Oficial del Estado, 1986; Codex Alimentarius Comision, 1969; Herlich, 1990). In the present work, the results for the physicochemical parameters were subjected to statistical analysis.

Block 2 presents significant direct relationships of the following groups of variables: (i) free acidity, lactone and total acidity, and where the Pearson correlation coefficient averages from 0.9357 to 0.7412; and (ii) ash, ash alkalinity and conductivity, and where the Pearson correlation coefficient averages from 0.9989 to 0.9935. Such correlation coefficients emphasise the extreme similarity between these physicochemical parameters. This is not surprising, taking into consideration that total acidity is the result of the sum of both free and lactone acidities, and that ash and ash alkalinity are calculated by using electrical conductivity (Andrade, 1995; Andrade et al., 1999). Therefore, the measurements of total acidity and electrical conductivity are adequate for a regular analytical quality control, whereas the other well-correlated parameters can be determined on a less regular basis.

Block 2 presents RPCs until the sixth PCs, accounting for 83.30% of total variance. The first two RPCs express only 46.52% of total variance, PC1 (27.78%) and PC2 (18.74%), respectively (Figure 3a). The RPCA states that the first two PCs tend to distinguish the honey samples in terms of acidity (total, lactone and free), and ash, ash alkalinity and conductivity (Figure 3a). No significant differences are found in this data block in terms of production year and locality. Therefore, RPCs 3–6 were further investigated.

RPCs 3–6 account for 36.77% of total variance (data not shown). In these four PCs, the loadings of apparent sucrose and HMF are the most relevant. Apparent sucrose discriminates the year 1991 from the years 1992 and 1993, decreasing from 1.405 ± 0.496 to 0.963 ± 0.416 and 1.000 ± 0.421 ($p < 0.05$), respectively. There is no geographical discrimination in this block of data.

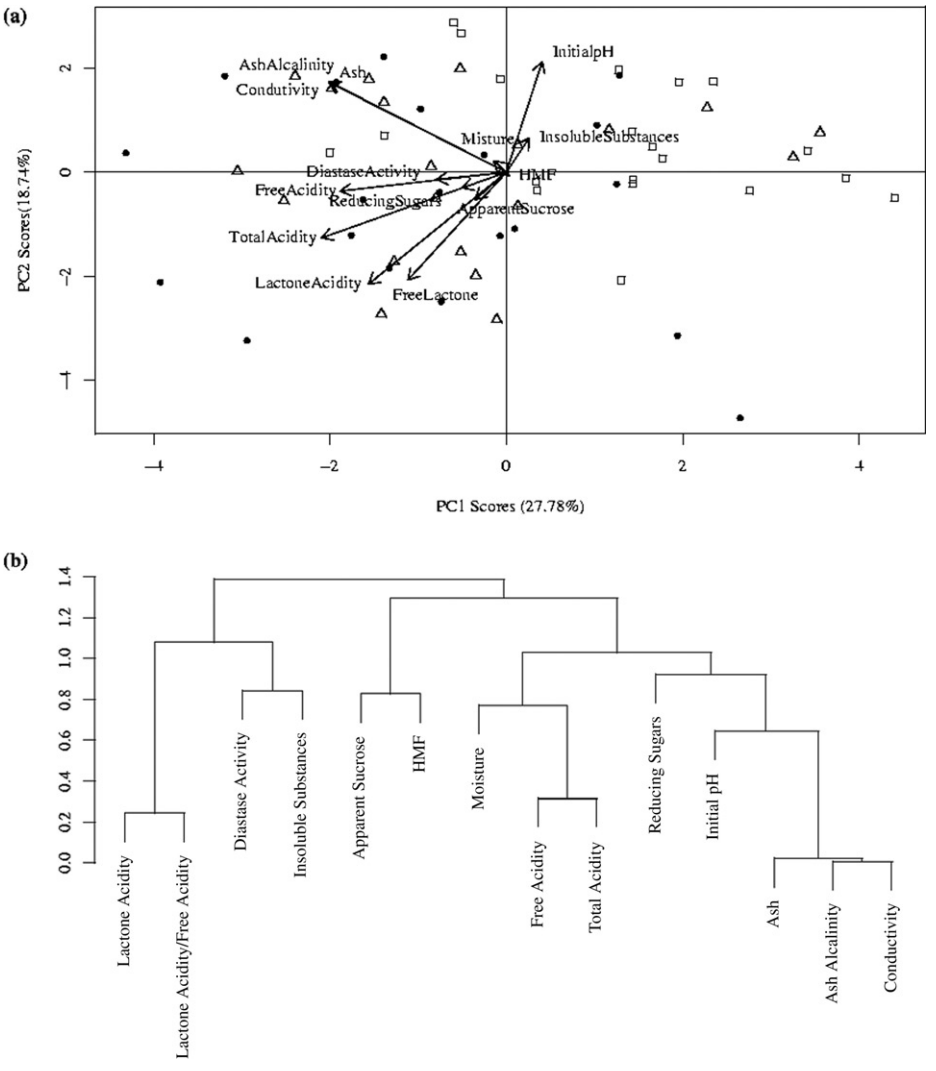

Figure 3. 'Serra da Lousã' honey physicochemical parameters PCA: (a) Gabriel plot of PC1 (27.78%) *vs.* PC2 (18.74%), with: ● 1991, □ 1992 and Δ 1993. (b) The first six PCs loading hierarchical clustering dendogram.

Figure 3(b) presents the hierarchical clustering dendogram of the first six RPCs loadings, which were organised into two major groups. In the first group, it is possible to observe: (i) lactone acidity and lactone acidity/free acidity; and (ii) diastase activity and insoluble substances. In the second group, it is possible to find strong relationships between: (i) ash, ash alkalinity and conductivity; and (ii) free acidity and total acidity; as already detected during Pearson correlation analysis. A third group is obtained with HMF and apparent sucrose. Inside this branch it is also possible to observe

that moisture content has loadings near to free acidity and total acidity, as well as reducing sugars and initial pH.

In conclusion, the sugars profile allows the discrimination between 'Serra da Lousã' and 'Terra Quente de Trás-os-Montes' honeys: 'Serra da Lousã' honeys do not contain sucrose, generally exhibit lower contents of turanose, trehalose and maltose and tend to present higher contents of fructose and glucose. The glycerol contents allowed discriminating samples from different localities into two distinct groups, with high and low glycerol contents. Glycerol and ethanol contents revealed to be independent of the sugars profile. No significant differences between the physicochemical parameters of the 'Serra da Lousã' honey from the 20 different locations were found.

The RPCA has proven to be an important statistical tool to help in the characterisation of 'Serra da Lousã' honey. Only by selecting the relevant variables on each PC was it possible to reduce the dimensionality in relation to the traditional PCA. Furthermore, the use of RPCA allowed for a better interpretation of the relationships between the honey constituents. The RPCA models obtained in this study and the results presented herein can be used as a database for the detection of adulteration in 'Serra da Lousã' honey.

3. Experimental

3.1. *Samples*

Erica sp. honey samples produced in the 'Serra da Lousã' region (Table 11) were provided and guaranteed by Direcção da Circunscrição Florestal de Coimbra (Portugal). Sixty samples were collected in July during three consecutive years (from 1991 to 1993), obtained by centrifugation and kept at 0°C until analysis, which occurred within 1 month

Table 11. Geographical origin of 'Serra da Lousã' heather honey samples.

Sample	Origin
1	Miranda do Corvo (Cruz Branca)
2	Góis (Alvares)
3	Vila Nova de Poiares (Alveite Grande)
4	Penela (S. João do Deserto)
5	Pampilhosa da Serra (Portela do Fojo)
6	Castanheira de Pêra (Alge)
7	Arganil (Piodão)
8	Arganil (Coja)
9	Arganil (Rochel)
10	Arganil (Mourão)
11	Arganil (Pombeiro da Beira)
12	Pedrógão Grande (Louriceira)
13	Pedrógão Grande (Romão)
14	Figueiró dos Vinhos (Campelos)
15	Figueiró dos Vinhos (Arraçais)
16	Lousã (Favariça)
17	Lousã (Cerdeira)
18	Lousã (Padrão)
19	Lousã (Foz de Arouce)
20	Lousã (Sarnadinhos)

after extraction from the hives by beekeepers. Samples were considered as heather honey by beekeepers based on their organoleptic properties.

Twenty samples of *Lavandula stoechas* L. honey were obtained from 'Terra Quente de Trás-os-Montes' (Vila Flor – Portugal) in 1991. The same sampling conditions of *Erica* sp. honey samples were applied.

3.2. *Sugars composition*

3.2.1. *Sample preparation*

Honey samples (5 g) were dissolved in 25 mL of redistilled water. The resultant solution was transferred to a 50 mL volumetric flask, which was made up with acetonitrile, and then passed through a Chromabond C18 column (500 mg). The purified solution was filtered (0.45 μm), degassed in an ultrasonic bath and 20 μl were analysed by HPLC.

3.2.2. *HPLC analysis*

Sugar separation was achieved with an analytical HPLC unit (LKB), using a Bondpack/Carbohydrate (300×4 mm) column. Elution was carried out at a solvent flow rate of 1.5 mL min^{-1}, isocratically, with acetonitrile/water (80/20) as the mobile phase. Detection was accomplished with a Gilson Refraction Index detector. The compounds in each sample were identified by comparing their retention times with those from authentic standards and quantified by external standard method.

3.3. *Primary normal alcohols determination*

Primary normal alcohols were determined by applying the modified Boehringer–Mannheim enzymatic method, as previously reported (Huidobro et al., 1994). Spectrophotometric determinations were performed at 340 nm and results were expressed as apparent ethanol contents.

3.4. *Glycerol determination*

Glycerol was determined spectrophotometrically at 365 nm, according to the modified Boehringer–Mannheim enzymatic method described previously (Huidobro et al., 1993).

3.5. *Physicochemical analysis*

Moisture, electrical conductivity, HMF, diastase activity, pH, acidity (free, lactone and total), reducing sugars, apparent sucrose and insoluble material were determined according to the European Community (Codex Alimentarius Comision, 1969), Portuguese (NP 1307 and 1309, 1976), Spanish (Boletin Oficial del Estado, 1986) and AOAC methods (Herlich, 1990). Total ash, soluble and insoluble ash, sulphated ash, and alkalinity of soluble, insoluble and total ash were determined as reported previously (Sancho, Muniategui, Huidobro, & Simal, 1992).

3.6. *Statistical analysis*

Statistical analysis and the RPCA algorithm were performed with R 2.1.1 for Linux, using the following packages: (i) classical multivariate analysis library (mva); (ii) main library of Venables and Ripley's (MASS); (iii) Harrell miscellaneous (Hmisc); and (iv) R-base packages ('R-Project R', http://www.r-project.org/∞).

Statistical analysis comprised the exploration of patterns and plausible data driven correlations between: (i) sugars profile; (ii) ethanol content; (iii) glycerol content and (iv) physicochemical parameters. The first step of the analysis involved the study of the Pearson correlation coefficients between the different honey components to explore the most evident interactions between constituents (Montegomery, 1991; Neter, Kutner, Natchtsheim, Wasserman, 1996). Thereafter, data blocks were submitted to RPCA. Data records presented in Tables 1–10 were organised in the following data blocks:

Block 1, sugars profile and ethanol and glycerol contents: fructose, glucose, sucrose, turanose, maltose, trehalose, isomaltose, raffinose, melibiose, melezitoze, total sugars, ethanol and glycerol.

Block 2, physicochemical parameters: moisture, ash, reducing sugars, apparent sucrose, diastase activity, HMF, pH, free acidity, lactone acidity, lactone acidity/free acidity, total acidity, insoluble material, ash alkalinity, conductivity (Andrade, 1995; Andrade et al., 1999).

3.6.1. *Relevant principal component analysis*

Principal component analysis (PCA) is a generalised methodology for pattern recognition in data analysis, where correspondences between variables are discovered by analysing the PC in terms of variance contribution (eigenvalues), variable correspondence inside each PC (eigenvectors – loadings analysis) and sample positions on the PC space (scores analysis) (Krazanowski, 1998). PCA reduces the dimensionality of a data set by the transformation of variables into a set of new variables (PCs), which compact the most important effects in the data. PCs are ordered in terms of the variance expression on the data set, where the PC_i expresses the i's largest variation for all PCs. The PC_i is obtained by maximising the projections of data points, so that it is orthogonal to the previous $i-1$ PCs (Jollife, 1986).

Generally, in data sets with well-structured variance, the most relevant variation is captured in the first few PCs. However, with less structured data, variance is spread over a larger number of PCs. In these cases, it is difficult for the traditional PCA to be interpreted and to make use of the reduced dimensionality to explain the variability of the data set. The RPCA only uses the statistically significant loadings on each principal component to try to overcome this difficulty. This not only allows for a better interpretation on how the different variables affect the data variability, but also helps to recognise which variables are most related by multivariate analysis.

3.6.2. *RPCA methodology*

The RPCA is a variation of the PCA with the objective of finding which variables are statistically relevant in each PC. When it is not possible to find relevant principal

components, this method uses all the variables to compute the PC. Figure 4 presents the RPCA algorithm flowsheet for better understanding of this technique.

The data blocks used in RPCA were pre-processed by auto-scaling (mean centering and scaling each variable's variance):

$$\hat{X} = \frac{X_i - \bar{X}_i}{\sigma(X_i)} \tag{1}$$

where $\hat{X}$ is the auto-scaled data, X_i the individual data value for the variable i, $\bar{X}_i$ the average value of the variable i, and $\sigma(X_i)$ the variance of the variable i. Auto-scaling is important when comparing data with different magnitudes and units, such as the one presented in this research.

The RPCA uses the singular value decomposition (SVD) (Alter, Brown, & Botstein, 2000; Holter et al., 2000) of the auto-scaled data matrix to compute the PCs:

$$[\hat{X}] = [U][S][V]^{\mathrm{T}} \tag{2}$$

where $[U]$ is the right singular matrix, $[S]$ the singular values, and $[V]^{\mathrm{T}}$ the left singular matrix.

Principal component scores are obtained by multiplying the right singular ($[U]$) by the singular values ($[S]$), whereas the left singular represents the PCs loadings matrix ($[V]^{\mathrm{T}}$).

The relevant variables in the first PC1 are determined by a randomisation test (Figure 4). Loadings are proportional to the covariance matrix $[C]$, and therefore it can be estimated by:

$$[C] \propto ([U][S])^{\mathrm{T}}[\hat{X}] \tag{3}$$

By performing a randomisation, it is possible to determine different values of $[C]$, by randomly sampling the PC1 scores ($[U][S]$):

$$[C]^{\mathrm{rand}} = ([U][S])^{\mathrm{rand}}[\hat{X}] \tag{4}$$

Thereby, it is possible to access the statistical significance of each variable by estimating their loading's p-value. The p-value is determined by the number of randomisations that the module of estimated correlation values are above those originally determined by SVD:

$$p = \frac{\sum_{i=1}^{n_{\mathrm{rand}}} M\left(\left|[C]^{\mathrm{rand}}\right| > \left|[C]\right|\right)}{n_{\mathrm{rand}} + 1} \tag{5}$$

where p is the probability value, M is a binary function that takes the value 1 if $|[C]^{\mathrm{rand}}| > |[C]|$ is true; and 0 if false. That is, given a large number of randomisations (e.g. $n_{\mathrm{rand}} = 5000$), any relevant variable will present a small probability of difference between $[C]^{\mathrm{rand}}$ and $[C]$. Relevant PC variables were considered to present $p < 0.05$ (95.0% level of significance) (Figure 4).

Once it is known that variables are statistically relevant for PC1, the algorithm determines the first relevant principal component (RPC1) loadings and scores only with the statistically relevant variables by SVD:

$$[\hat{X}]_{\mathrm{RelVars,RPC1}} = [U][S][V]^{\mathrm{T}} \tag{6}$$

where $\hat{X}]_{\mathrm{RelVars,RPC1}}$ is the pre-processed dataset truncated to the relevant variables.

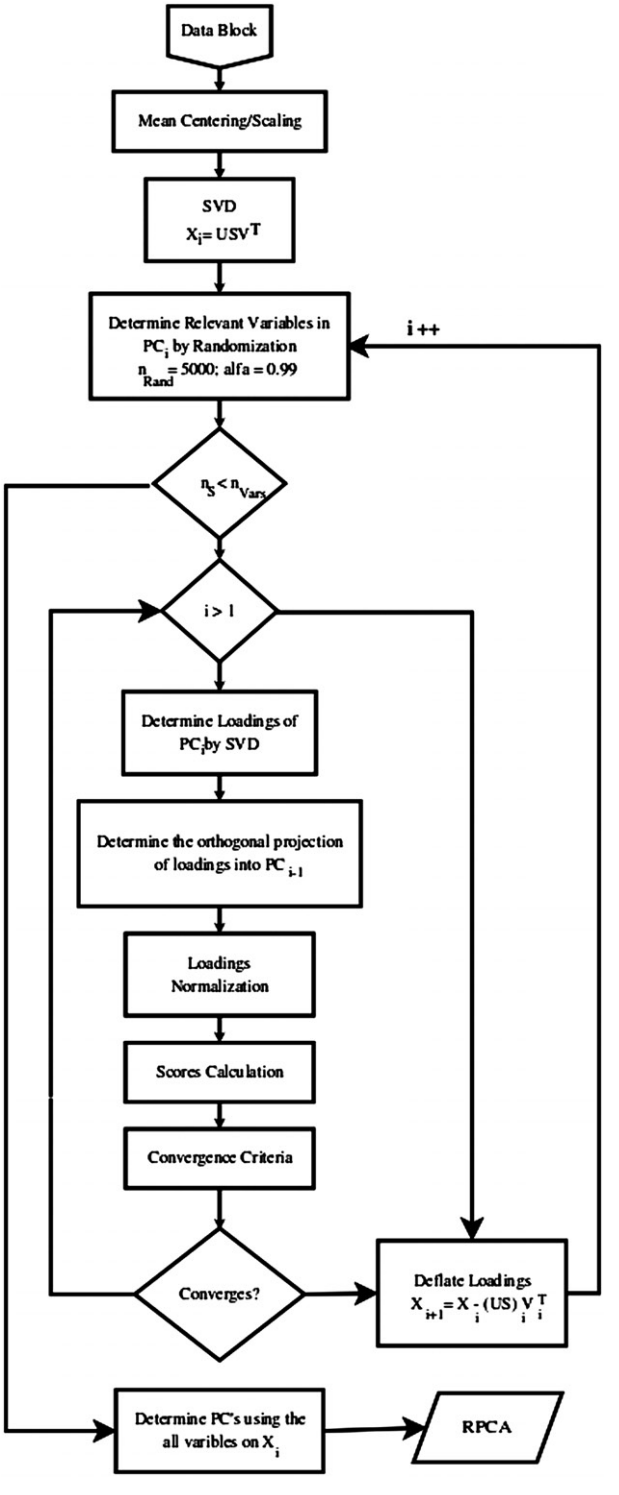

Figure 4. Relevant principal component algorithm.

After computing the RPC1, it is possible to determine the next PC, by extracting from the original data the RPC1 direction:

$$[\hat{X}]_{i+1} = [\hat{X}]_{i+1} - ([U][S])_i[V]^{\mathrm{T}} \tag{7}$$

where $\lfloor \hat{X} \rfloor_{i+1}$ is the data used to estimate the new RPC, $\lfloor \hat{X} \rfloor_i$ the original data used for the determination of RPC1, $([U][S])_i$ the scores, and $[V]_i^{\mathrm{T}}$ the loadings from RPC1, respectively (Figure 4).

The algorithm estimates the next RPC by applying an SVD to $\lfloor \hat{X} \rfloor_{i+1}$ (Figure 4). Similarly, the RPC2 is estimated by a randomisation test to access the loadings statistical significance. After determining the relevant variables of PC2, the RPC2 is estimated by an iterative fashion (Figure 4). The SVD using the relevant variables is used as a first approximation to the final RPC2. Orthogonality between PCs is only possible if the number of singular values is inferior to the number of relevant variables, which, otherwise, makes it impossible to solve the linear set of algebraic equations to obtain an orthogonal PC. If this condition is satisfied, the RPC2 is estimated by the following of these sequential steps:

(1) Determination of loadings by using an SVD transformation:

$$[V]^{\mathrm{T}} = \left(([U][S])([U][S])^{\mathrm{T}}\right)^{-1}([U][S])[\hat{X}]_{\mathrm{RelVars},i+1} \tag{8}$$

(2) Determination of the orthogonal projection of the RPC2 loadings with RPC1:

$$[V]^{\mathrm{T}}_{\mathrm{proj}} = [V]^{\mathrm{T}} - [V]^{\mathrm{T}}[O] \tag{9}$$

where $[O]$ is obtained by:

$$[O] = [V]^{\mathrm{T}}([V][V]^{\mathrm{T}})^{-1}[V] \tag{10}$$

(3) Normalisation of the estimated loadings:

$$[V]^{\mathrm{T}}_{\mathrm{norm}} = \frac{[V]^{\mathrm{T}}}{|[V]^{\mathrm{T}}|} \tag{11}$$

(4) Determination of the new estimated scores:

$$([U][S])_{i+1} = [V]^{\mathrm{T}}_{\mathrm{norm}}[\widehat{X}]_{\mathrm{RelVars},i+1} \tag{12}$$

(5) Computing the error between the estimated scores in steps $i+1$ and i:

$$\varepsilon = ([U][S])_{i+1} - ([U][S])_i \tag{13}$$

Convergence was considered when an error (ε) of 5×10^{-15} was obtained. If no convergence is reached, the algorithm re-calculates all the steps 1–4, until it reaches convergence or the maximum number of iterations is attained (e.g. 1500, Figure 4). Once convergence is found, the RPCA algorithm continues to search for relevant PCs by extracting from the original data the RPC2 direction to estimate RPC3 recursively, repeating all the presented steps in Equations (8)–(13) (Figure 4). If no convergence is found, or if the number of singular values is larger then the number of relevant variables, the algorithm proceeds with the calculation of the PCs by the normal SVD methodology

using all the existing variables, as presented in Equation (2) using $[\hat{X}]_i$. Once these two conditions are met, it is not possible to find any other relevant PC on the dataset, meaning that PCs extracted after these criteria are considered random and should not be used for sample characterisation. Under these circumstances, relationships between honey constituents were only derived using hierarchical clustering analysis of the eucledian distance on the relevant PCs.

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