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HPLC-fluorimetric method for analysis of amino acids in products of the hive (honey and bee-pollen)

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Abstract

An optimization of the OPA method has made feasible separation and quantification of 23 amino acids, which include 5 infrequently searched for. Detection limits ranged from 0.24 to 10.1 pmol in honey and from 29.1 to 0.42 pmol in bee-pollen; reproducibility (C.V.) ranged from 5.3% to 20.4%; recoveries were above 78.8%. Forty monovarietal honey samples from ilex, oak, heather and chestnut-tree were analyzed for their free amino acid profiles. α-Aminoadipic acid and homoserine are reported for the first time in honeys. Thirty-two samples of Spanish bee-pollen, made of a majority of pellets from *Cistus Ladanifer* (67.1%) and *Echium plantagineum* (8.9%), were analyzed for their free and total amino acid profiles. Free γ-aminobutyric acid was extensively found with an average of 0.53 mg/g, while Hser and Orn were infrequent. Manually separated monofloral pellets from *Cistus ladanifer* and *Echium plantagineum* were analyzed for their free amino acid contents (including proline): 32.46 and 21.87 mg/g for the former and 22.18 and 12.23 mg/g for the latter. In contrast, the total amino acid percentage (on a dry weight basis) was 13.95% for *Cistus ladanifer* and 32.22% for *Echium plantagineum*.

Keywords: Amino acids; Honey; Bee-pollen

1. Introduction

Pollen of plants is by far the most important source of proteins and free amino acids for the bees. Of the hive products, royal jelly, bee brood and the bee-collected pollen contain high amounts of them; honey and bee venom contain much smaller quantities, while beeswax none (Crane, 1990a, 1990b). Certain amino acids are derived from the bees and are common to many honeys (White, 1976), while others originate in parts of the plants different from pollen, like nectar and honeydew. Proline is the major free amino acid both for honey and pollen, with amounts that may surpass widely half of the total free amino acid content. In the case of honey it is mainly contributed by the bees and is originated in the pollen they consume early in life (Crane, 1990a, 1990b; Louveaux, 1985). Although several pathways are now known, all the processes that give rise to such a great amount in bee-pollen still remain unclear (Serra Bonvehí, Gómez Pajuelo, & Gonell Galindo, 1986; Standifer, McCaughey, Dixon, Gilliam, & Loper, 1980). Anyway, amounts of protein material in honey are always very low and varying, with average values of 0.1% for total proteins and 0.3% for free amino acids (Bosi & Battaglini, 1978; Davies & Harris, 1982). In contrast, bee-collected pollen shows much higher content of protein, but it varies greatly according to the plant

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source, ranging from 7% (pine) to 35% (date palm) (Auclair & Jamieson, 1948; Witherell, 1975). Most of the amino acid content is in the bound form (Grünfeld, Vincent, & Bagnara, 1989; Stanley & Linskens, 1974), so that total free amino acid amounts may be as low as one fifth of it (Grünfeld et al., 1989; Serra Bonvehı & Escolà Jordà, 1997).

So far, interest in knowledge of the amino acids profile for both products of the hive has centered on three fields: first, as a potential tool for the botanical or even the geographical differentiation of honeys (Cometto, Faye, Di Paola Naranjo, Rubio, & Aldao, 2003; Conte, Miorini, Giomo, Bertacco, & Zironi, 1998; Cotte et al., 2004; Hermosın, Chicón, & Cabezudo, 2003; Iglesias, De Lorenzo, Polo M.C, Martın-Álvarez, & Pueyo, 2004; Pérez-Arquillué & Herrera, 1987); secondly, from a nutritional point of view, in the case of pollen, as a source of proteins or essential amino acids (Abreu, 1992; Bell et al., 1983), and third, for quality control as indicator of freshness and adequacy of the drying process and storage of pollen, based on the content of a few free amino acids (Muniategui, Sancho, Huidobro, & Simal, 1991; Serra Bonvehí & Escolà Jordà, 1997; Serra Bonvehí et al., 1986).

When the HPLC technique appeared, reagents were tested to obtain UV-absorbing derivatives of amino acids to be measured either spectrophotometrically or fluorimetrically. Reagents employed for this purpose are *o*-phthaldialdehyde (OPA), dansyl chloride (DANS-Cl), 9-fluorenilmethylchloroformate (9-FMOC), or phenylisothiocyanate (PTIC). Each has pros and cons.

DANS-Cl reacts both with primary and secondary amines, but high temperature and a long time are required. The derivatives are rather unstable and each amino acid yields more than one, which complicates the chromatogram. PTIC also reacts with primary and secondary amines, yielding stable and UV-absorbing derivatives, without interfering by-products and detection limits of picomoles. However, successive drying steps are required, which makes it a time consuming and a poorly reproducible method when the operator is not trained. A known commercial application is available, but common laboratory material and a conventional chromatograph have been employed by some authors (Heinrikson & Meredith, 1984), even in honey (Pérez-Arquillué & Herrera, 1987), to avoid costs derived from it.

Both OPA and FMOC react to yield highly fluorescent derivatives and, thus, they are advocated when a high sensitivity is needed. FMOC reacts with primary and secondary amines, yielding stable compounds, but formation of fluorenilmethylchloroformate, an alcoholic derivative, is generally reported as a major drawback as it may spoil the chromatogram. Pre-column reaction of amino acids with OPA, in the presence of 2-mercaptoethanol (MCE), proceeds at room temperature yielding isoindolic derivatives in a quick and simple reaction (Fig. 1). However, secondary amino groups, such as those of proline and hydroxyproline do not react. Some derivatives are unstable, which makes an appropriate control crucial of both the times of reaction and injection (Hanczkó & Molnár-Perl, 2003).

Ideally, an analytical method for amino acids should comply with the following requirements: short time of analysis, high sensitivity (picomoles), linear response and, finally, stable and rapidly forming derivatives without any interfering artifact. In addition, the reagent must react with both the amino- and the imino-groups and preferably with a pre-column reaction to avoid a costly post-column chamber. In our work the OPA/MCE reagent was finally selected since, due to its high sensitivity (picomoles), minor amino acids would also be quantified. The employment of a chromatographic system equipped with a pre-column chamber permitted an automatic control of both the reaction and injection times. Measurement of proline and hydroxyproline was the only limitation, but an individual official method was employed to report on the abundant proline. Research on hydroxyproline was sacrificed – no particular attention seems to be paid to it in the literature.

A main goal of this work was the optimization of the OPA-HPLC method to identify and quantify as many amino acids as possible, with a special interest in a few of them not yet reported in the products of the hive. A particular application of it was made to the investigation of the free amino acid content of four monovarietal Spanish dark honeys, two of them from honeydew, and on the free and total amino acid profiles of Spanish bee-pollen – knowledge of the latter is restricted to its free fraction (Serra Bonvehí & Escolà Jordà, 1997) but not to the bound. Regarding the honeys studied, preliminary results pointed out a correlation between amino acid profiles and botanical origin. This is of particular interest for honeydew honeys, in our case from ilex

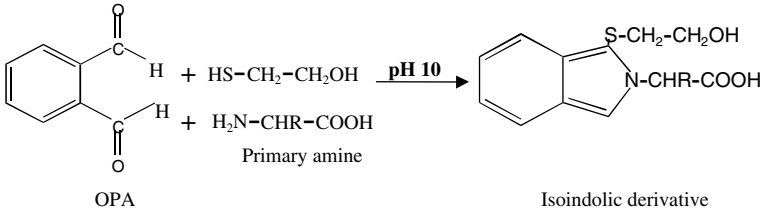

Fig. 1. Reaction of *o*-phthaldialdehyde and 2-mercaptoethanol with primary amines.

and oak, since neither the melissopalinological analysis – absence of pollen – nor the physico-chemical one enables their differentiation. Concerning the bee-pollen, most of the free and total amino acids are found in the inner and resistant layer of the pollen grain, the so-called sporopollenine. A procedure for disruption of it was investigated so as to ensure that the extraction proceeded adequately.

2. Materials and methods

2.1. Reagents and chemicals

A kit of high purity L-amino acids, from SIGMA, consisted of 1 g of each of the following 22 standards: alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), cystine (Cys_2), glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), hydroxyproline (Hyp), isoleucine (Ileu), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). The following individual standards were also supplied separately by SIGMA: taurine (Tau), γ-aminobutyric (Gaba), aminoisobutyric acid, ornithine (Orn), α-aminoadipic acid, 3,5-dibromotyrosine (I.S.), norleucine (Nleu) and homoserine (Hser). Individual solutions in methanol:water (50:50) were prepared at the desired concentration. The internal standard 3,5-dibromotyrosine was prepared at 4×10^{-4} M for honey and 10^{-2} M for bee-pollen. Working solutions were prepared in a 0.4 M borate buffer of pH 10 from the multicomponent standard solution within the calibrate range of each amino acid. Internal standard was added to the working solutions to reach the final desired concentration of 4×10^{-5} M for honey and 10^{-4} M for bee-pollen. The mixture for derivatization was prepared in a 25 ml volumetric flask by dissolving 500 mg of reagent OPA (Merck, for fluorescence analysis) in 22.5 ml of ethanol (Lichrosolv® from Merck, for liquid chromatography) and making it to volume with 0,4 M borate buffer of pH 10. 400 μl of 2-mercaptoethanol (Sigma) was added, eventually. A previous filtration through a 0.45 μm membrane (Millipore) for organic solutions is recommended. Ionic-exchange resins DOWEX® 50WX8-200 (Sigma) were required only for honeys and prepared as indicated below. The rest of reagents and chemicals were either HPLC or reagent analysis quality, as needed.

2.2. Materials

Amino acids were quantified with a Varian chromatographic system, which consisted of a 9012Q pump, 9100 autoinjector and 9075 fluorescence detector. Separation was carried out in a Waters Nova-Pack® reverse phase C18 column, 4 μm particle size, 150×3.9 mm i.d. A specific Nova-Pack® guard column was placed between the autoinjector and column. All the chromatographic information was reprocessed in a Star Workstation (ver. 4.5) supplied by Varian. A glass column of 1.5×30 cm and a rotavapor (Büchi R-114) was employed for the honey preparation. A sonicator (Microson XL2007) and a homogenizer (Polytron PT 10–35 from Kinematica) were employed for the preparation of the bee-pollen.

2.3. Samples

Throughout years 1991–1998, a total of 40 monovarietal honey samples from ilex, oak, heather and chestnut-tree (10 of each) were supplied only by beekeepers (ilex and oak) or both by beekeepers and specialized stores (heather and chestnut-tree). Microscopic analysis, included pollen and honeydew elements, confirmed their origin. Nevertheless, in the case of honeydew honeys (ilex and oak) certification of monovarietal botanical origin was made by the beekeepers, as no chemical or biological test is available for it. All were kept frozen before analysis. Processing of the samples before injection of the extracts was limited by the fact that keeping them in the fridge made the borate buffer crystallize.

A total of 32 samples of Spanish bee-pollen, collected throughout years 1999–2001, were supplied both by beekeepers and specialized stores. Palynological composition was: 72% *Cistaceae* (of which, 92.7% *Cistus ladanifer*); 4% *Boraginaceae*, *Papilonaceae*, *Asteraceae*, and *Fagaceae*, each; 1% *Rosaceae* and *Ericaceae*, each; and a 10% of other botanical families. After microscopic analysis, samples were kept frozen before preparation for chromatographic analysis.

2.4. Samples preparation

Honey, *for free amino acids* (Fig. 2). For the separation of sugars a DOWEX® 50WX8-200 resin is conditioned as follows. Let 3 g of resin stand in 400 ml of distilled water for 2 h, then shake it and introduce the suspension in a glass column at a rapid speed. Continue flow of water until the column height remains stable and the rinsing water becomes neutral. [Regeneration of resin must be carried out after use with successive portions of 10 ml of 2 N HCl and ultrapure water until the reaction of the rinsing liquid becomes neutral]. 1.0 ml of 3,5-dibromotyrosine is added to 1.0 g of honey and dissolved in 10 ml of phosphate buffer pH 2.12, then added to the top of the column and sugars are eluted with 3×10 ml of water. Then amino acids are eluted with 15 ml of 7 N NH_4OH and 10 ml of ultrapure water. The collected solution is vacuum evaporated to dryness at a temperature below 50 °C and redissolved in 10 ml of borate buffer of pH 10.

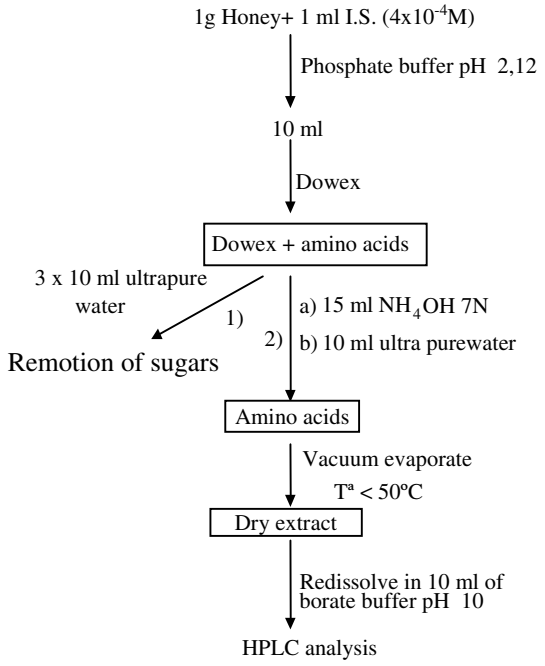

Fig. 2. Clean-up procedure and extraction of free amino acids from honey before chromatography.

Bee-pollen, for free amino acids (Fig. 3). Take some 10–15 g of bee-pollen loads in a mortar. Weight 250–300 mg, add 25 ml of 80% ethanol, disperse it for 30 s with the help of the sonicator and homogenize with the Polytron for 2 min. Shake for 15 min, centrifuge 5 min at 2000 rpm and decant the supernatant. Repeat extraction over the sediment twice with two more 25 ml quantities of 80% ethanol. The ethanolic extract solution is vacuum evaporated to dryness at a temperature below 40 °C and redissolved in a volumetric flask with ultrapure water to 50 ml. Filter through a 0.45 μm membrane (Millipore) and keep it frozen until analysis. Take 2.0 ml extracts, add 50 μl of 10^{-2}M I.S. and make with water to 5.0 ml in a volumetric flask.

Bee-pollen, for total amino acids (Fig. 3). Weigh some 100 mg of bee-pollen loads into a pyrex screw-cap tube. Add 3 ml of ultrapure water and sonicate for 40 s to a complete dispersion of loads. Add 3 ml of 12 M HCl and remove the air in the tube with a nitrogen stream. Put the cap on it tightly and heat at 110 °C for 22 h. Then let the tube cool at room temperature, filter its content through a common filter paper and rinse it up to collection of some 25–30 ml. Neutralize the acid hydrolysate partially to pH 4–6, make up to 50 ml volume with ultrapure water, filter through a 0.45 μm membrane (Millipore) and keep it frozen until analysis. Take 2.0 ml extracts, add 50 μl of 10^{-2}M I.S. and make with water to 5.0 ml in a volumetric flask.

Honey and bee-pollen for proline. An official method of the Spanish regulation (Presidencia del Gobierno,

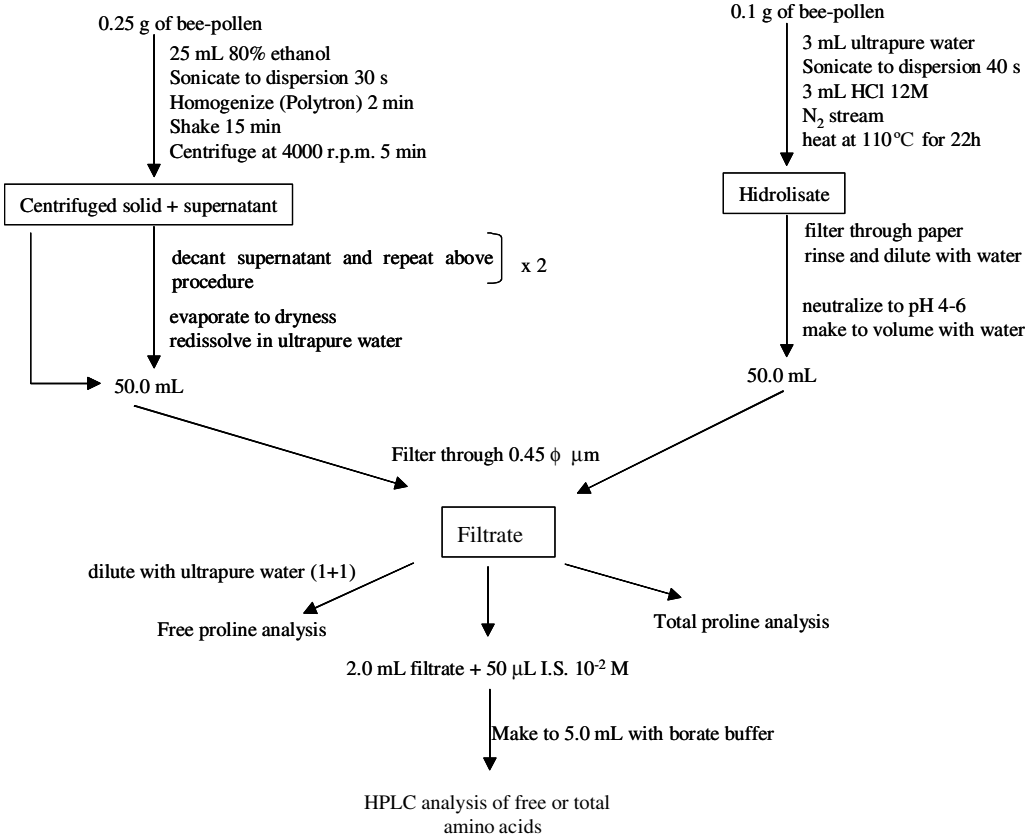

Fig. 3. Preparation of bee-pollen for free and total amino acids analysis.

1986) was employed based on the colorimetric measurement of reaction with acidic ninhydrin. An adaptation to bee-pollen was made; for the free proline, a dilution of the extract to 50% is made with ultrapure water; for the total proline no dilution was necessary. The samples were analyzed in triplicate.

2.5. HPLC analysis

The sample extracts (0.5 ml for honey and 1.0 ml for bee-pollen) are submitted to an automatic precolumn reaction using 100 μl of derivatizing reagent. The chromatographic conditions are as follows: flow 0.1 ml/min until minute 3 and then 1.5 ml/min; volume of injection 10 μl for honey and 20 μl for bee-pollen; and solvents, A, sodium phosphate buffer (10 mM, pH 7.3): methanol: tetrahydrofurane (80:19:1) and B, sodium phosphate buffer (10 mM, pH 7.3): methanol (20:80). The gradient consists of: 100% A during 3.5 min, 0–15% of B in A for 6 min, 15% B isocratically for 5 min, 15–30% of B for 5 min, 30–40% of B for 4 min, and 40–80% of B for 12 min. Fluorimetric detection is carried out using excitation and emission wavelengths of 340 and 426 nm, respectively.

3. Results and discussion

3.1. Sample preparation

Honey. A removal of the sugars must be accomplished. The Dowex 50WX8 (H^+), a strongly acidic ion-exchange resin, has been ordinarily employed for this purpose (Bouseta, Scheirman, & Collin, 1996; Gilbert, Shepherd, Wallwork, & Harris, 1981). Honey dissolved in acidic medium is retained in the column while sugars are eluted by successive portions of pure or acidified water (Bosi & Battaglini, 1978), phosphate buffer of pH 2.12 (Bouseta et al., 1996) or a diluted HCl (Pirini, Conte, Francioso, & Lercker, 1992). Different options were tested by us, the best results being attained for ultrapure water as other eluants were observed to leave impurities that interfered in the chromatogram.

Bee-pollen. Most of the free and bound amino acids of pollen are inside the so-called exine (sporopollenine), a most resistant cellulose material provided with pores. Following a natural process, when conditions of moisture are suited the intine breaks, as a result of hydration, and emerges through the pores. Some authors (Serra Bonvehí & Escolà Jordà, 1997) have profited from this natural process and proposed suspension of pollen in water for more than one hour so that the protein material can be extracted; others have tested hydroalcoholic and diluted HCl solutions. Experiments were carried out by us to assess and optimize the process of extraction with tools that included microscopic observation of pollen. 80% Ethanol gave rise to a clear extract as proteins were precipitated, but no ejection of the intine material was noticeable at the optic microscope. HCl 0.1 M offered results very similar to water. Next, a combination of a mechanical action and the three above extractants were tested. Breaking of the resistant exine was attempted separately with a grinder and a sonicator. Microscopic observation revealed an extensive, although uncompleted, rupture in both cases, but the need for the temperature to be maintained under control to avoid interconversion of amino acids was a limiting factor and prevented us from the necessary intensive application. [No cooling system was tried out for it.] The Polytron homogenizer proved more effective, although not all the grains were observed broken. It operates inside the extraction tube, with an ice cooling system and over a small quantity of sample. A combination of sonicator, homogenizer and 80% ethanol as extractant was finally agreed. It speeds up the process, since a rapid rupture occurs and the subsequent drying of the extract proceeds at low temperature and in a short time.

3.2. HPLC Method

General. Separation and quantification of 23 amino acids in a chromatogram of 35 min has been accomplished (Fig. 4). Uncommon amino acids such as Orn and Gaba were also included as their presence was reported in some honeys (Bouseta et al., 1996; Speer & Montag, 1986). Nleu has been extensively employed as an internal standard (I.S.) by many authors (Bouseta et al., 1996; Pirini et al., 1992). However, possible traces of this amino acid have been pointed out in a few honeys, which violates one of the rules for an I.S. The search for a compound that complies with these rules made us test for a number of amino acids. α-aminoisobutyric acid was found to elute together with Tau in a single peak; carnosine did not resolve completely and coeluted with Arg; α- aminoadipic acid and Hser were decided to be included as compounds to be searched for rather than as internal standards – the former appeared too early in the chromatogram. No reference was found in the literature on the presence of 3,5-dibromotyrosine and in our study it proved to comply with conditions for an I.S.

As known, previously, amino acids Asn and Gln could not be properly quantified by the Autoanalyzer device in honeys as they coeluted together with Ser and Thr, respectively (Davies & Harris, 1982) and much more recent studies quantify together Asp + Asn and Glu + Gln (Conte et al., 1998), As*n* + Ser (Hermosín et al., 2003) and Thr + Ala and Trp + Orn, (Cometto et al., 2003). All these disadvantages are overcome by our method.

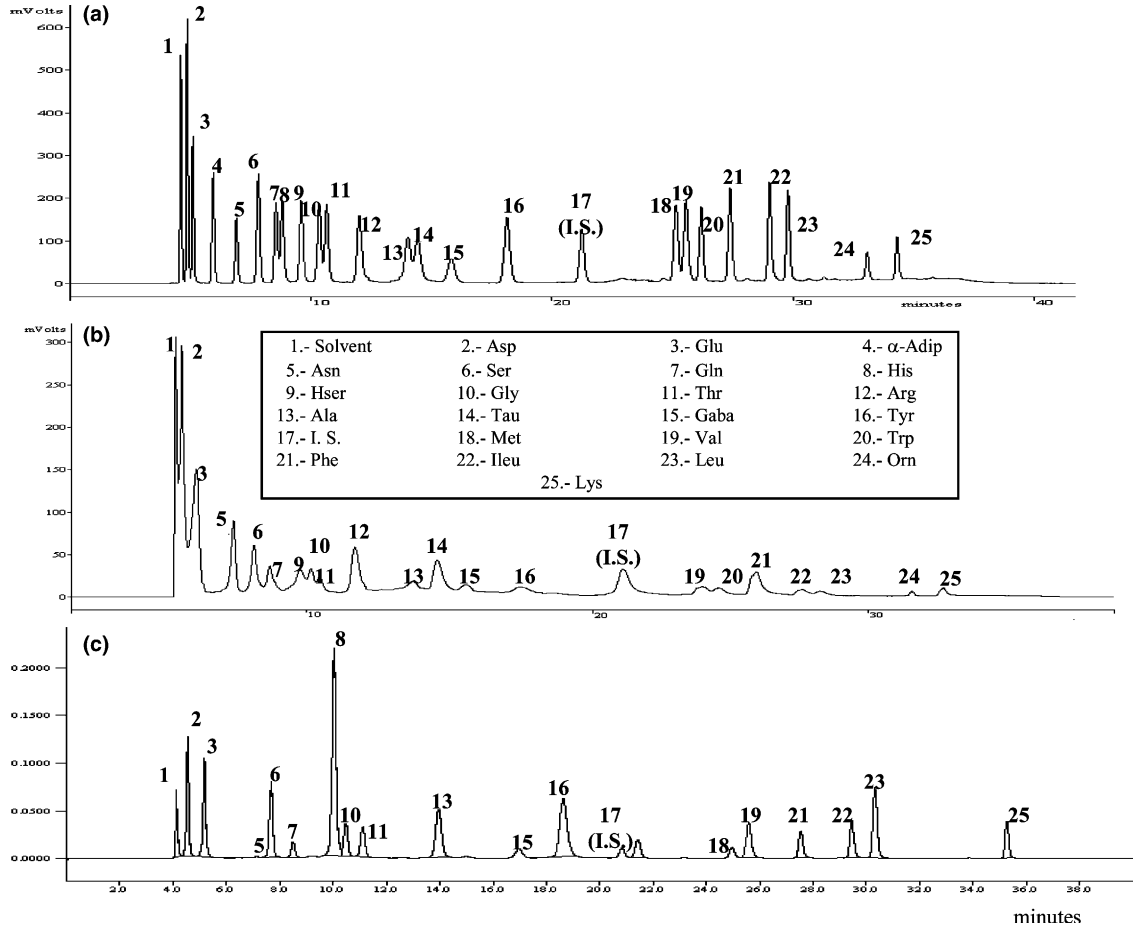

Fig. 4. Chromatograms of amino acids in a solution of standards (a), in a honey from ilex (b) and in a sample of bee-pollen (c).

Table 1
Validation of methods for honey and bee-pollen

	Honey			Bee-pollen					
				Free amino aicds			Total amino acids		
	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min
Detection limit (pmol)	10.1	1.73	0.24	29.1	3.19	0.43	5.60	1.74	0.42
Reproducibility (C.V., %)	16.7	12.71	9.5	20.4	12.2	5.35	16.7	10.6	5.3
Recovery (%)	109.0	94.8	78.8	99.0	89.0	80.0	92.0	86.0	80.0

Regarding bee-pollen, the method proved to be suitable, although the above mentioned scarcity of studies prevents us from a critical comment. It should be stated that Trp cannot be measured for total amino acids owing to the acidic hydrolysis process.

Table 1 shows the analytical validation of the methods for honey and bee-pollen. Detection limits were established according to Glaser, Foerst, Mckee, Quave, and Budde, 1981. Seven replicate solutions of amino acids, with concentrations ranging from two to five times the estimated detection limit, were submitted to the above analytical protocol, which in the case of honeys included also the purification step through ionic-exchange resins. Standard deviation (SD) was calculated from the replicates and the detection limit (DL) from the equation: DL = 3.707 SD.

Precision of method was established separately for standards, honey and bee-pollen as follows. Multicomponent solutions of standards were employed for the first case at two levels (10 pmol and 1 nmol). For honey, two samples with very distinct levels of amino acids were selected. For bee-pollen, a single level was tested. In any case, CV (%) was derived from seven replicates. Likewise, the values of recovery for honey and bee-pollen ($n = 7$) were established at two levels.

As shown in Table 1, in several cases the CV surpass the value of 10% generally reported by other authors.

This can be accounted for by the lower levels employed by us for this study.

Free amino acid content of the honeys assayed. Should proline be included, all 24 amino acids searched for were found in at least one of the 40 samples. As generally recognized, proline is a major one with average contents of 800–850 mg/kg for the four honeys studied. Table 2 shows that proline fills up half of the free amino acid profiles for ilex and heather honeys. The next most abundant are Gaba for chestnut-tree (22.4%) and oak (7.2%); Asn (9.4%) for ilex and Phe (12.9%) for heather. The abundance of Phe in nectar honeys has been reported at length (Bosi & Battaglini, 1978; Conte et al., 1998). In a study (Pirini et al., 1992) on six kinds of honeys (acacia, citrus fruit, chestnut-tree, rhododendron, rosemary, and lime-tree) Arg was found only in chestnut-tree, so that it was indicated as a possible parameter for discrimination. Our study, although a preliminary work, reveals the presence of Arg in all four kinds of honeys, although the highest amounts were found for chestnut-tree. Concerning Trp, most authors (Bosi & Battaglini, 1978; Pirini et al., 1992) report only traces of it in all the honeys analyzed so far. Our study reveals important amounts of it, with average values of 41 mg/kg for ilex and as high as 615 mg/kg for chestnut-tree (see Table 2). Up to the present, α-aminoadipic acid

Table 2
Average (%) amino acid profiles of the four types of honeys

Amino acid (%)	Honey			
	Ilex	Oak	Chestnut-tree	Heather
Pro	**49.5**	**37.6**	**29.6**	**49.6**
Asp	2.6	4.1	2.9	3.0
Glu	4.1	4.7	2.8	0.6
α-Adip	0.1	0.2	0.1	0.0
Asn	**9.4**	6.2	3.3	3.1
Ser	1.3	1.4	1.5	1.4
Gln	5.1	3.4	2.5	1.7
His	0.8	3.2	3.5	0.6
Hser	0.3	0.2	0.5	0.5
Gly	0.6	0.3	0.4	0.7
Thr	0.5	0.9	0.5	0.8
Arg	1.6	2.5	2.7	1.3
Ala	2.7	2.3	1.5	2.2
Tau	4.3	3.1	2.1	4.1
Gaba	2.2	**7.2**	**22.4**	3.4
Tyr	1.6	1.1	7.6	1.1
Met	0.8	1.1	1.0	1.5
Val	0.5	0.5	0.6	0.5
Trp	1.3	4.4	2.7	4.4
Phe	2.7	6.4	3.4	**12.9**
Ileu	0.4	0.8	3.6	1.1
Leu	0.2	0.4	0.4	0.5
Orn	0.2	1.9	1.7	3.7
Lys	0.9	1.5	0.9	1.0

Bold characters highlight the higher percentages for each.

Table 3
Average content (mg/g pollen) of free and total amino acids of 32 samples analyzed and of *C. ladanifer* and *E. plantagineum* loads manually separated

Amino acid (mg/g bee-pollen)	Free amino acid			Total amino acid		
	Bee-pollen	*Cistus*	*Echium*	Bee-pollen	*Cistus*	*Echium*
Asp	0.40 ± 0.21	0.41 ± 0.02	0.57 ± 0.03	15.10 ± 3.68	13.52 ± 0.90	32.34 ± 0.69
Glu	0.25 ± 0.15	0.26 ± 0.04	0.97 ± 0.11	17.88 ± 1.98	16.09 ± 0.48	16.36 ± 0.59
α-Adip	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.
Asn	0.52 ± 0.48	0.53 ± 0.06	2.00 ± 0.01	3.87 ± 1.03	3.43 ± 0.27	5.68 ± 0.07
Ser	0.60 ± 0.31	0.55 ± 0.36	0.30 ± 0.10	2.74 ± 0.77	3.05 ± 0.08	2.41 ± 0.12
Gln	0.60 ± 0.48	0.81 ± 0.38	0.17 ± 0.12	5.91 ± 2.53	4.98 ± 0.07	14.23 ± 0.02
His	0.74 ± 0.67	0.31 ± 0.01	0.10 ± 0.00	6.84 ± 7.15	3.78 ± 0.30	44.90 ± 2.47
Hser	0.03 ± 0.08	0.14 ± 0.01	< D.L.	–	–	–
Gly	0.21 ± 0.19	0.19 ± 0.10	0.31 ± 0.07	6.40 ± 1.09	5.69 ± 0.04	11.20 ± 0.74
Thr	0.25 ± 0.21	0.25 ± 0.03	0.83 ± 0.25	4.17 ± 0.61	4.21 ± 0.23	5.50 ± 0.10
Arg	2.48 ± 1.61	2.32 ± 1.14	1.49 ± 0.35	5.03 ± 1.49	4.26 ± 0.26	8.27 ± 0.54
Ala	0.82 ± 0.65	0.94 ± 0.12	0.97 ± 0.17	10.68 ± 1.09	9.66 ± 0.27	13.31 ± 0.11
Tau	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.
Gaba	0.35 ± 0.33	0.70 ± 0.20	< D.L.	–	–	–
Tyr	0.32 ± 0.15	0.31 ± 0.15	0.31 ± 0.05	7.43 ± 3.13	5.71 ± 0.30	37.56 ± 1.60
Met	0.29 ± 0.30	0.24 ± 0.06	0.13 ± 0.01	4.10 ± 1.53	3.69 ± 0.12	7.23 ± 0.62
Val	0.21 ± 0.11	0.25 ± 0.05	0.11 ± 0.01	7.26 ± 1.94	5.54 ± 0.11	15.76 ± 0.52
Trp	0.09 ± 0.10	< D.L.	< D.L.	–	–	–
Phe	0.75 ± 0.40	0.73 ± 0.24	0.19 ± 0.14	9.65 ± 2.16	8.80 ± 0.09	27.16 ± 1.01
Ileu	0.51 ± 0.55	0.49 ± 0.02	0.60 ± 0.26	9.22 ± 2.01	8.57 ± 0.34	15.98 ± 0.03
Leu	0.91 ± 0.43	0.40 ± 0.21	0.65 ± 0.37	10.81 ± 1.65	8.51 ± 0.52	16.87 ± 1.53
Orn	0.08 ± 0.21	0.79 ± 0.21	< D.L.	–	–	–
Lys	0.26 ± 0.29	0.38 ± 0.24	0.24 ± 0.19	10.97 ± 1.94	9.88 ± 0.23	37.11 ± 1.01
Pro	20.27 ± 3.82	21.87 ± 0.63	12.23 ± 1.13	22.88 ± 3.53	23.91 ± 0.60	19.39 ± 0.78
Total	30.9 ± 4.49	32.5 ± 0.76	22.2 ± 2.56	161.2 ± 23.70	143.3 ± 0.62	331.3 ± 0.11

and Hser had never been reported in honeys. In our study, the former was found in 16 samples, 7 of which came from chestnut-tree, and the latter in 25 samples.

Concerning the total content of free amino acids, our results are very similar to those reported (Bosi & Battaglini, 1978; Gilbert et al., 1981; Speer & Montag, 1986) for heather and honeydew honeys, with some 1500–2000 mg/kg. Chestnut-tree honeys show an average of 2764 mg/kg, mostly owing to their high content of Gaba.

Free and total amino acids content for Spanish bee-pollen (Table 3). Our results for free amino acids agree with the only scientific work published (Serra Bonvehı & Escolà Jordà, 1997) for Spanish bee-pollen – the major species was *Cistus ladanifer* too. Twenty two free amino acids were found and, as with the honey, proline was a major one with an average of 20.27 mg/g pollen. Free Gaba was extensively found with an average of 0.53 mg/g, while Hser and Orn were infrequent (22% and 16% of samples, respectively). Regarding the total amino acids content, proline is also a major component although Glu (average 15.10 mg/g pollen) reaches very similar figures. As the percentage of *Cistus ladanifer* falls and that of *Echium plantagineum* rises, free amino acids percentage reflects a slight drop while that for total amino acids rises up to a larger extent. An important increase in the free proline content is the reason for the apparent decrease of the free amino acids fraction, which corresponded to samples collected towards the end of spring. The fact that different proportions of loads from both species influence the free and total amino acids content was investigated. Loads from three different samples were manually separated for *Cistus ladanifer* and *Echium plantagineum*, so that three monofloral bee-pollen samples of each species were analyzed. Table 3 shows the average free amino acids and proline for *Cistus ladanifer* loads were of 32.46 and 21.87 mg/g, while for *Echium plantagineum* loads were of 22.18 and 12.23 mg/g, respectively. In contrast, the total amino acids percentage (on a dry weight basis) was 13.95% for *Cistus ladanifer* and 32.22% for *Echium plantagineum*. Since proline is contributed by the bee (Louveaux, 1985), the variable contribution results in a different balance of proline to total amino acids.

Finally, the different content of total Hys and Tyr should be highlighted (44.90% and 37.56% for *Echium plantagineum*, against 3.78% and 5.71% for *Cistus ladanifer*, respectively). Other amino acids such as Lys and Asp also show significant differences.

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