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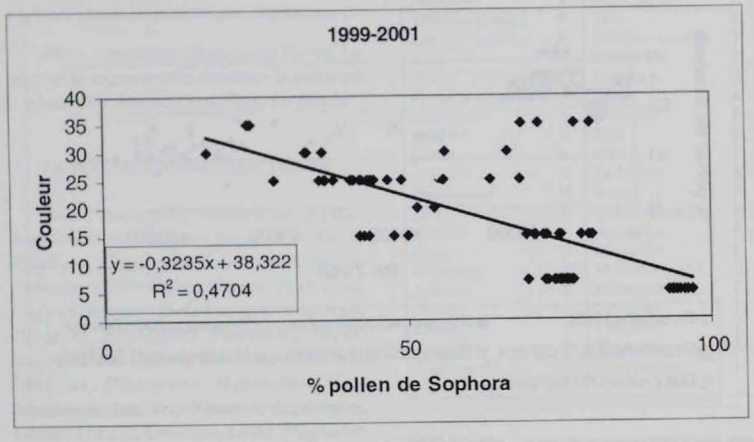

FIGURE 5. Corrélation Couleur/Pourcentage en pollen de Sophora

Polen 13:373-377(2003)

PHENOLIC COMPOUNDS OF *ECHIUM PLANTAGINEUM* L. POLLEN

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(Manuscrito recibido el 26 de Noviembre de 2002, aceptado el 30 de Junio de 2003)

SUMMARY: Presently humans consume of bee (*Apis mellifera* L.) collected pollen is a fast growing market, which interest is based in some empirical evidences of health properties, with scarce scientific support. In this direction we studied pollen phenolics profile of *Echium plantagineum* L. as specific botanical markers, finding it as a source of pollen rare flavonoids and anthocyanins. To achieve this, we used HPLC-DAD and HPLC-DAD/MS techniques. Our results revealed that *E. plantagineum* pollen blue colour is due to the presence of an anthocyanin petunidin-3-rhamnoglucoside, probably petunidin-3-rutinoside. The most abundant flavonoid was kaempferol-3-glucoside-7-rhamnoside and the second was kaempferol-3-(6''-malonylglucoside)-7-rhamnoside, which has never been referred in the literature.
KEY WORDS: *Echium plantagineum*, bee, *Apis mellifera* L., pollen, HPLC, anthocyanins, flavonoids.

RESUMEN: Presentemente el consumen humano de polen recohido por las abejas (*Apis mellifera* L.) es un mercado de rapido crescimiento, con base en algunas evidencias empíricas de beneficios para la salud, pero sin suporte cientifico. En esta dirección nosotros estudiamos lo perfil fenolico del polen de *Echium plantagineum* L. como marcadores botánicos especificos, encontrandolo como una fuente de un raro flavonoide e antocianas. Para lograr esto, nosotros usamos HPLC-DAD y técnicas de HPLC-DAD/MS. Nuestros resultados revelaram que el colour azul del polen de *E. plantagineum* es debido a la presencia de la antociana petunidin-3-rhamnoglucoside, probablemente el petunidin-3-rutinoside. El flavonoid más abundante era kaempferol-3-glucoside-7-rhamnoside y el segundo era el Kaempferol-3-(6''-malonylglucoside)-7-rhamnoside que nunca fuera referido en la literatura.
PALABRAS CLAVE: *Echium plantagineum* L., abeja, *Apis mellifera* L., polen, HPLC, anthocyanins, flavonoids.

INTRODUCTION

In spite of being native to Europe, *E. plantagineum* L. is, nowadays, world-wide distributed (CARRASCO, 2001; SOMMERVILLE, 2001). Although its non-edible character, it represents a major source of food for bees, mainly its pollen but nectar as well. In fact, the *E. plantagineum*'s pollen appears to be particularly attractive to bees (SOMMERVILLE,

2001). As bee collected pollen is achieving more interest from consumers, the *Echium sp.* pollen has also increasingly been included in human diet which emphasizes the importance of studying its nutritional value. Among commercial bee collected pollen, the presence of the almost unique blue pellets of *Echium* species makes it richer and more attractive.

Frequently, pollen is considered to have special stimulatory proprieties in animals but unfortunately the scientific information available is scarce. CHAUVIN (1968) and STANLEY & LINSKENS (1974) mentioned several scientific research works, which refer the exceptional weight gain in calf, piglets and broilers fed with pollen supplements. KRETSCHMAR & BAUMANN (1999) found very high levels of caffeine and teofiline in *Citrus*' pollen. In the same direction, KHRIPACH *et al.* (2000) showed that *Brassica napus* and *Vicia faba* pollen had important levels of steroidal hormones, which might have potential effects on animals after consuming. Nevertheless, the most important aspect referenced in literature is that pollen has very strong positive effects in the treatment of various prostate illnesses, including cancer (BUCK *et al.*, 1989; BRUNETON, 1993).

Some of those effects may be attributed, to pollen antiradical and antioxidant activities, since they present high levels of carotens, xanthophylls, and flavonoids (STANLEY & LINSKENS, 1974; WIERMANN & GUBATZ, 1992), all related with anticancer proprieties (BENAVENTE-GARCÍA *et al.*, 1997; BRITTON, 2002; HEINONEN, 2002).

Concerning pollen flavonoids, they are already fairly well studied, mainly after Wiermann works (WIERMANN, 1968; WIERMANN & VIETH, 1983), and more recently, by high-performance liquid chromatography (HPLC) techniques, for searching specific genetic markers, making these compounds of real importance to bee products market (MEURER *et al.*, 1988; FERRERES *et al.*, 1993; GIL *et al.*, 1995; CAMPOS, 1996; MARTOS *et al.*, 2000).

Although its lipidic content is rather low (about 2%) (SOMMERVILLE, 2001), the *Echium* sp. pollen is promptly collected by bees, contradicting previous works that related pollen attractiveness with its kit lipid content (mainly odoriferous carotenoid derivates). So, pollen poor in carotenoids would not be collect by bees but these flowers would be visited for its nectar reservoirs (LEPAGE & BLOCH 1968; DOBSON, 1988, 1991).

Regarding protein content, it is already known that plant species which produce pollen with low protein levels, apparently with no special interest for honeybees that still visited these flowers as they may present odorant cues. However, this is not the ca se of *E. plantagineum,* which has been considered one of the five best protein quantity (35% protein content) and quality sources in Australia (SOMMERVILLE, 2001).

The high attractiveness of *Echium* sp. pollen to bees can not rely on its protein content, because bees seem not to recognise it easily and thus, other factors such as the visual impact (colour pigments) could be involved.

Many flowers have visual cues based on ultraviolet light that appear to be of great value to attract insects. As *E. plantagineum* produces pollen with low levels of essential oils, its great attractiveness should be associated with flower and pollen colours and its ultraviolet-visible bee's perception. CARRASCO *et al.*, (2001), photographed *Echium*

sp. flowers with special filters, simulating bee perception, and found that the flower tube colour greatly contrasts with the background. In addition, some almost imperceptible spots in corolla were revealed. These authors concluded that this species developed a very efficient strategy for bee attraction based on colour, which is due to the presence of anthocyanins (HARBORNE & MABRY, 1982).

In the present work, we studied the flavonoids and anthocyanic fractions of *E. plantagineum*'s pollen towards a complete characterisation of the species pollen profile using HPLC-DAS-MS.

MATERIAL AND METHODS

In this study, fresh pollen of *E. plantagineum* from the Northern of Portugal was used. The pollen flavonoids and anthocyanins were identified through HPLC-DAD and HPLC/MS techniques:

For extraction of such compounds bee collected pollen was added to 70% aqueous methanol, agitated, centrifuged at 12.000 g and passed through a 45 mm mesh.

Acid extraction was performed adding bee collected pollen to 70% aqueous acidic (5% formic acid) methanol, centrifuged at 12.000 g and filtered through a 45 mm mesh.

Merck LichroCART 250-4 and LiChrospher 100RP-18 (5 mm) columns were used. The positive Mass Spectroscopy (MS) mode was used to anthocyanins and negative MS mode to flavonoids.

For the HPLC, the solvent system was: Solution A (methanol) and solution B (1% formic acid in water); the flow rate was 1mL/min and was established the following elution gradient: (0 minutes – 30% Sol A; 20 minutes – 40% Sol A; 25 minutes – 50% Sol A; 40 minutes – 50% Sol A and 45 minutes – 70% Sol A).

For saponification, 450mL of extract were added to 50mL of NaOH 4N, placed in a closed nitrogen atmosphere for 1.5 hour. Afterwards, 20 mL of this solution was directly injected on HPLC.

Partial acid hydrolyses was also performed by adding 50 mL of 12N HCl to 950 mL of the extract refereed above, during 60 hours at room temperature. After this period 20 mL were directly injected on HPLC. Sequential hydrolysis was carried out each 30 min at 75ºC and 85ºC, and 20 mL samples were directly injected on HPLC.

RESULTS AND DISCUSSION

The chromatographic profiles obtained from bee collected *E. plantagineum* pollen extracts presented as blue pigment the anthocyanin petunidin-3-rhamnoglucoside, probably petunidin-3-rutinoside, showing absorvance peaks at 275 and 529 nm and a shoulder at 347 nm which revealed the presence of two sugars (Fig. 1). Mass Spectroscopy (MS) analysis indicated the presence of rhamnose and glucose.

The most abundant flavonoid was the kaempferol-3-glucoside-7-rhamnoside. Using MS with aid of a partial acid hydrolysis it was possible to find the connection point of sugars. A second flavonoid was identified, the kaempferol-3-(6"-malonylglucoside)-7-rhamnoside, not yet referred in the literature. In a previous study of *E. plantagineum* pollen it was considered to be kaempferol-3-glucoside (CAMPOS, 1996).

This blue pigment, although rare in pollen, might be responsible for the great attractiveness of this pollen to bees, as it seems to be very easy for them to detect it. But we can't reject the possibility of this pollen having important sensitive elements for bees. In Australia, SOMERVILLE (2001), considered *E. plantigineum* pollen as one of the 5 richest pollens harvested by bees in relation to protein content (35%), but there are other more attractive pollens with much less protein.

The phenolic profile of *E. plantagineum* pollen obtained in this work seems to be of great interest and presents several practical application, such as genetic markers in taxonomic and botanical studies, and so in human consumption pollen.

These results are in agreement with previous studies (WIERMANN & VIETH, 1983) which consider kaempferol more abundant than quercetin, in pollen of several dicotyledonous species. Since bees easily detect

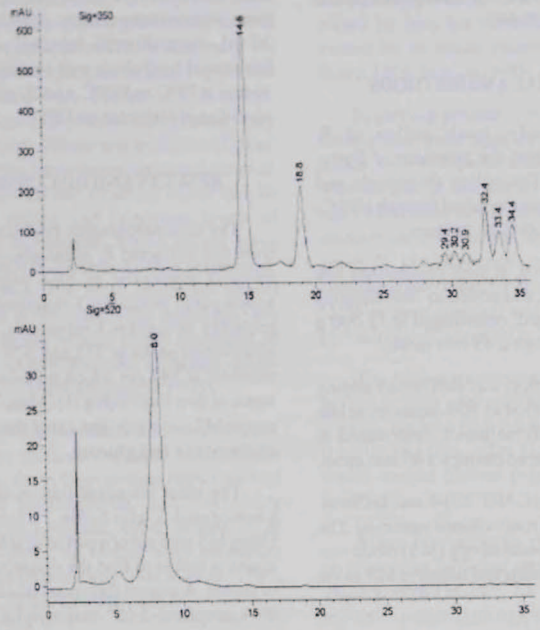

FIGURE 1. HPLC chromatograms (detection at 350 and 520 nm) of flavonoids and anthocyanins extracts of *E. plantagineum* bee collected pollen (tR 8.1min) petunidin-3-rhamnoglucoside; (tR 14,9 min) kaempferol-3-glucoside-7-rhamnoside, (tR 18,8 min) kaempferol-3-(6"-malonylglucoside)-7-rhamnoside.

the founded phenolics, rare in pollen, they might be reponsible for the great attractiveness of these insects by this pollen.

ACKNOWLEDGEMENTS

The first author acknowledge the financing provided by FCT Grant SFRH/BD/7049/2001.

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