Biodiversity Journal, 2019, 10 (4): 329–336 https://doi.org/10.31396/Biodiv.Jour.2019.10.4.329.336

Melissopalinological study of Sicilian honey by morphological and molecoular approach

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ABSTRACT With the Legislative Decree n.179 of 21 May 2004 on the unifloral honey's characteristics and with the CEE regulation 2081/92 of DOP denomination (from protect origin), scientific investigations in the agri-food sector started to occupy a prominent position with increasing importance. This area of investigation has been considerably deepened, becoming fundamental in order to consider this term valid, as it is also fundamental for investigation on production chain and denomination, made on various types of products. The morpho-genetic characterization analysis of pollen from honeys, made in the present work, lands itself well to this purpose; with the aim of ascertaining the validity of the wording of "unifloral" applied to some products from the beekeeping industry. Since the implications to which the investigations in the agri-food sector lead to cover not only the purely scientific-agronomic, but also the legal field, it follows the importance of an increasingly scientifically accurate methodology that also allows a faster processing of the sample. The protocol commonly used for pollen characterization of honeys is based on the visual recognition of the pollen present in the sample, their count and statistical analysis of the data obtained. Our method, using Real Time PCR technology, allows a qualitative and quantitative analysis of the pollen species inherent in the sample, thus allowing a fast and accurate analysis of the data that lends itself well to assist the classical research based on visual recognition of pollen.

KEY WORDS Honey; pollen; agri-food sector; DOP; PCR.

Received 11.07.2019; accepted 21.10.2019; published online 11.12.2019

INTRODUCTION

The Legislative Decree n. 179 of 21 May 2004 cites the characteristics of unifloral honey. These must have precise chemical-physical parameters (content of sugars, water, insoluble substances).

It also sets the minimum purity values to consider unifloral honey. In Italy, the Community Directive 74/409 of 22/7/74, which regulates the marketing of honey, defines the general criteria of composition and also provides for the possibility of declaring the botanical origin of honey, without offering the necessary means to identify the unifloral honeys.

The aim of the study is to be able to perform an evaluation, both through microscopic analysis and through biomolecular techniques, of the quantity and type of pollen granules present in the various commercialized unifloral honeys, in order to detect any case of fraud.

For this reason we have tried to optimize a multidisciplinary survey system for honey certification.

This type of investigation initially envisaged a phase of microscopic observation, aimed at the

study and recognition of the pollen morphologies among the various floral species through the study of the exine, the outermost wall of the pollen grains.

Generally mature pollen grains have variable dimensions and are covered by a layer of lipids and carotenoids which facilitate their adhesion on the stigma surface. The pollen has a protective layer composed of two walls: intine and exine.

The intine is constituted by polysaccharides such as cellulose and hemicellulose, the exine is constituted by Sporopollenin.

The presence of structures and ornamentations on the outermost portion of the exine allows to attribute a certain pollen morphology to a well-defined botanical species.

MATERIAL AND METHODS

The present study was carried out in the palynological and melissopalinological field, through the analysis of pollen granules of Mediterranean floral species and predominantly unifloral honeys (Pignatti, 1982; Accorti et al., 1986; Persano Oddo et al., 1991, 1995; Serra Bonvehí & Granados, 1993; Franck et al., 2000; Jerkovic et al., 2008; Lolli et al., 2008; Attenzio et al., 2016; Rodopoulou et al., 2017; Tariba et al., 2018; Can et al., 2015; Mannina et al., 2015; Marengo et al., 2017).

The palynological recognition consists in the analysis of the pollens that are extracted by washing the anthers through the use of ethyl ether, $(C_2H_5)_2O$.

The pollens that have been analyzed through this technique belong to some of the main botanical families used for the production of honey (*Citrus* spp., *Eucalyptus* sp., *Rosmarinus officinalis*, *Acacia* sp., *Heliantus annuus*).

Initially, microscopic observations were made without the use of coloring agents to analyze the pollen grains in their natural color which may change according to the botanical family.

In order to make the palynological recognition more efficient, we also created a colored preparation with fuchsin.

Fuchsin (0.1% alcohol solution) is prepared adding 100 mL of Ethanol (Et-OH) per 0.1g of basic fuchsin.

The color with the fuchsin makes more evident the morphological characteristics of the pollen grain, exine in particular, by observation under an optical microscope.

Observation without fuchsin

We proceed with the collection, in the field, of flowers and inflorescences with anthers loaded with pollen grains which present an entomophilous pollination.

After that, we proceeded to remove the anthers from the rest of the flower. Once removed, we wash with ethyl ether (1–2 ml) inside a porcelain capsule, to allow the release of the pollen grains, which will settle on the bottom.

At the end, let the ethyl ether, contained in the porcelain capsule, be decanted under a chemical hood. As soon as the ethyl ether has evaporated completely, we can see that the pollen grains have settled on the bottom of the capsule.

If there are some floral parts in the porcelain capsule, detached after washing with ethyl ether, it is necessary to remove them.

Subsequently, 150 µl of distilled H_2O will be taken with a micro pipette and poured into the porcelain capsule containing the pollen, making “up and down” with the same pipette in order to bring as many pollen grains as possible into solution. Now, 50 µl of distilled H_2O containing the pollen will be taken and placed on a glovebox.

We will wait for the evaporation of the distilled H_2O (at room temperature or at a temperature not higher than 40 °C) to proceed to the observation of the pollen grains with an optical microscope (Figs. 1–4).

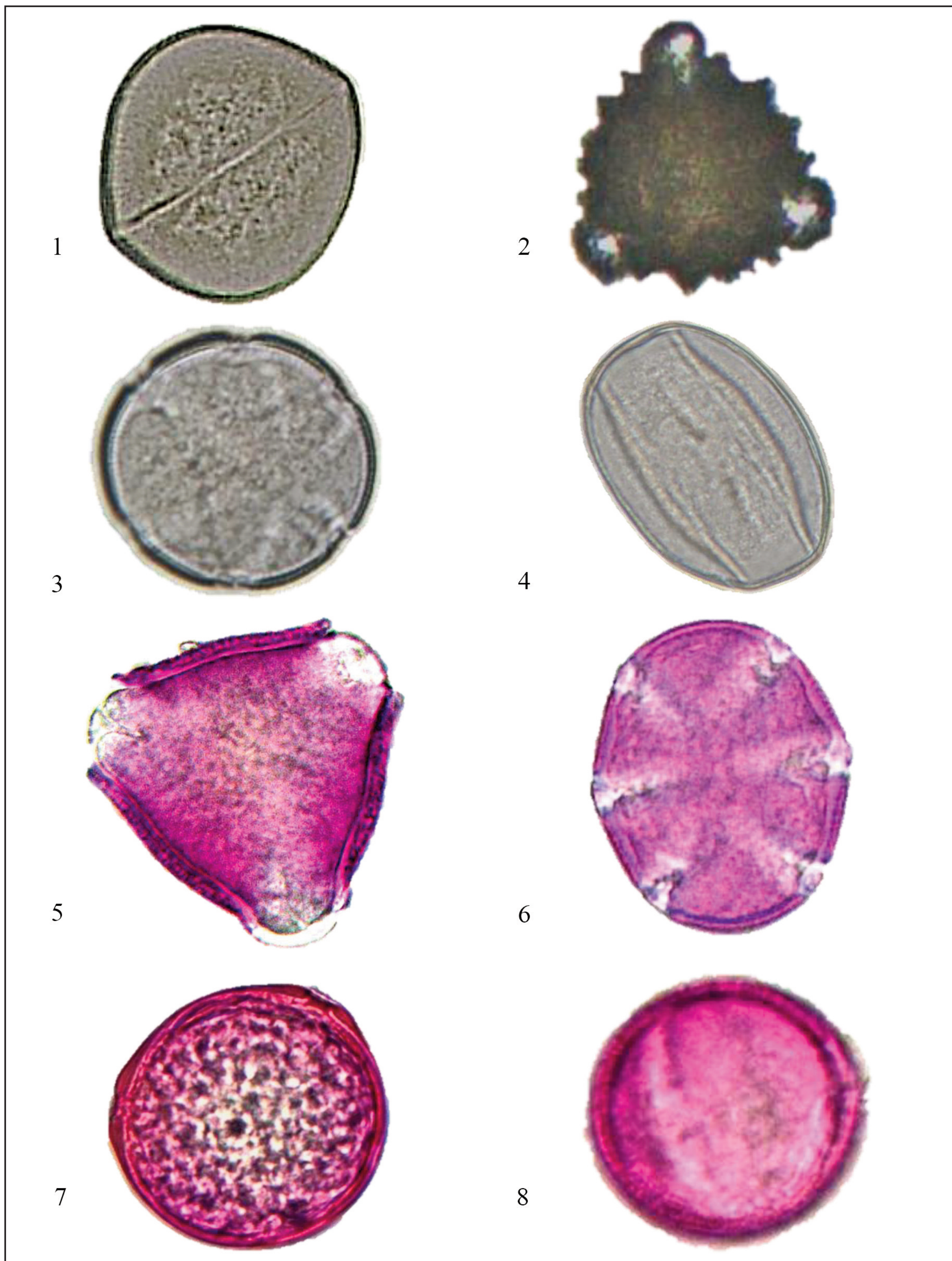
Observation with fuchsin

First of all the glycerine gelatin is liquefied through a thermostatic bath or in a microwave oven for no more than 5 seconds.

Since the pollens have a different affinity towards fuchsin, it is advisable to prepare a set of glycerine gelatin containing the basic fuchsin in different concentrations (from 0.2 to 1.5 ml of fuchsin every 10 ml of glycerine gelatin).

Once the basic fuchsin has been added, we stir it to obtain a homogeneous mixture.

For the creation of the colored preparation the same steps were carried out for the non-colored preparation, adding glycerine gelatin containing



Figures 1–4. Pollen granules without fuchsin: *Asphodelus* sp. (Fig. 1), *Carduus galactites* (Fig. 2), *Rosmarinus officinalis* (Fig. 3), *Prunus dulcis* (Fig. 4). Figures 5, 6. Pollen granules with fuchsin: *Prunus dulcis* (Fig. 5), *Rosmarinus officinalis* (Fig. 6), *Ailanthus altissima* (Fig. 7), *Citrus aurantium* (Fig. 8).

fuchsin before observation under a microscope.

15 µl of gelatine containing the fuchsin are taken and poured onto the glove containing the pollen.

At this point a coverslip will be applied to distribute uniformly the gelatin containing fuchsin.

Once the gelatine containing the fuchsin has solidified we can proceed to the observation under an optical microscope (Figs. 5–8).

All the steps were made working with a chemical hood, due to the presence of volatile toxic substances.

Melissopalinological recognition

After the recognition of the pollen morphology of the harvested floral species, through the optical microscope, it was possible to proceed with the melissopalinological analysis through the extraction of the pollen grains from various commercialized honey and to the microscopic observation, in order to perform an initial analysis compositional (Loveaux et al., 1978).

We take 10 g of honey and place it inside a 50 ml Falcon. Subsequently, 40 ml of distilled water are poured into the same Falcon, stirred to mix and place the Falcon in a thermostatic bath for about 30–40 minutes (depending on the honey density) in order to completely melt the honey in water.

After this step, the Falcon containing our sample will be centrifuged at 15,000 rpm for 15 minutes to separate the solid phase, containing the pollen grains, from the sugars and from the waxes that are present in honey.

After centrifugation the liquid phase is removed and it is possible to observe the sediment, consisting of the pollen grains, on the bottom of our Falcon.

To make sure that most of the sugars and waxes are effectively removed, which could interfere with both microscopic observation and DNA extraction, our sediment is removed, transferred to a 15 ml Falcon and suspended again in 10 ml of distilled water. Then, the steps of the thermostatic bath and centrifugation at 15,000 rmp for 15 minutes will be repeated.

Now it is possible to remove the liquid phase again. The pollen grains on the bottom are suspended in 100 µl of distilled H_2O and placed on a glove slide and viewed through a microscope.

The glycerine colored gelatine with fuchsin is applied to the glovebox to make the morphology of the pollens more evident.

Through microscopic analysis it is possible to make a first analysis of the pollen composition of the honey we are analyzing.

DNA extraction

To carry out a more precise analysis, we extracted the DNA from the pollen grains using special DNA extraction kits from plant tissues, in our case the E.Z.N.A. Plant.

Before starting to extract DNA it is important to make sure to dilute the DNA Wash Buffer with 60 ml of 100% ethanol. It is also necessary to prepare two 1.5 ml eppendorf, one containing distilled H2O and the other containing the Elution Buffer, which must be heated to 65 °C.

At this point we can proceed with DNA extraction.

We take 10 g of honey, place it in a 50 ml Falcon and dilute it with distilled water in a total volume of 50 ml. Centrifuge at 15,000 rpm for 15 minutes to precipitate the pollen component.

We remove the liquid being careful not to transport the sediment. Now resuspend the sediment in a 2 ml eppendorf, adding 1 ml of distilled water.

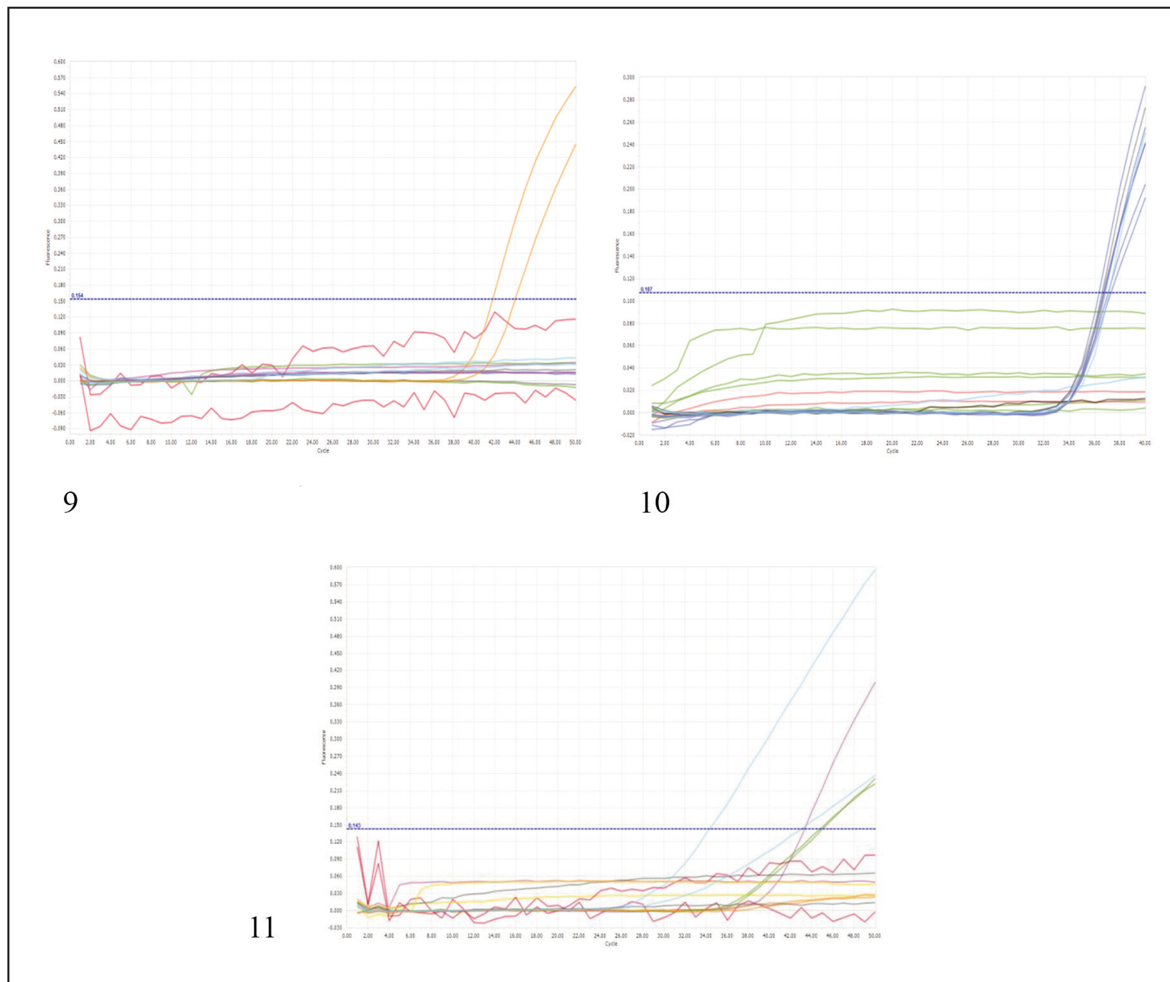
Centrifuge again for 15 minutes at 15,000 rpm. Now we can remove the liquid through a micropipette. In our eppendorf only the sediment containing the pollen grains from which we want to extract the DNA will remain.

Add 600 µl of Buffer P1 to our eppendorf containing the pollen sample and stir it in order to obtain a homogeneous mixture. Incubate at 65 °C for 10 minutes. After incubation, add 140 µl of Buffer P2, stir to homogenate and then centrifuge at 10,000 rpm for 10 minutes.

Transfer all the lysate, taking care not to transfer the insoluble part deposited on the bottom, into a new 1.5 ml eppendorf and add 0.7 volumes of isopropanol, stirring to mix. Centrifuge at 14,000 rpm for 2 minutes to precipitate DNA.

Remove the supernatant, taking care not to transport the sedimented DNA. Add 300 µl of distilled water that we previously heated to 65 °C and 4 µl of RNase A and stir to homogenate.

Add 150 µl of Buffer P3 and 300 µl of 100% ethanol and stir to mix. During this phase precipitates may form. In this case, do "up and down" with the micropipette for 10–15 times in order to homogenate and resuspend the precipitate.



Figures 9–11. Real time PCR on DNA extracted from honeys declared unifloral. DNA extracted from sunflower honey. The only amplified curves are those of the sunflower-specific genes (Fig. 9). DNA extracted from *Eucalyptus* honey. In addition to the specific *Eucalyptus* genes (first two top curves), genes specific to other botanical species have been amplified (Fig. 10). DNA extracted from orange honey. As for *Eucalyptus*, both the specific orange gene and the genes of other botanical species (Fig. 11) have been amplified.

Transfer the entire sample into a column in a 2 ml collection tube and centrifuge for 1 minute at 10,000 rpm.

Remove the collection tube containing the filtrate and use a new one.

Add 650 µl of DNA Wash Buffer into the column and centrifuge at 10,000 rpm for another minute. Remove the filtrate and repeat the previous step again. Centrifuge the column, to dry it completely.

Once dried, we will remove the collection tube and transfer the column to a 1.5 ml eppendorf.

Add 50 µl of Elution Buffer heated to 65 °C and centrifuge for 1 minute at 10,000 rpm.

Repeat this last step without removing the filtrate.

Now we can quantify the extracted DNA to know the concentration of DNA in our sample and its purity.

In Table 1 it is possible to observe the results of DNA quantification.

Real Time Pcr

Real time PCR was performed on the DNA extracted from the pollen grains collected, during the melissopalinological analysis, from honey of eucalyptus, sunflower and citrus declared unifloral.

For each DNA sample to be analyzed, through real time PCR, different mixes are prepared, each containing specific primers for a specific botanical species (Laube et al., 2010; Schievano et al., 2013; Cilia et al., 2018).

Real time PCR was performed in a total volume of 25 µl.

12.5 µl of the TaqMan™ Universal PCR Master Mix, containing the Taq polymerase, magnesium ions, reaction buffer and dNTP, are taken and inserted into a 0.2 ml eppendorf.

Then we will take 0.5 µl of each of the two primers (forward and reverse) and 0.5 µl of the probe and insert them into our eppendorf. Finally add 6 µl of distilled water.

The amplifications were carried out with the following protocol: 56 °C for 3 minutes, 95 °C for 10 minutes followed by 40 cycles of 15 seconds at 95 °C and 45 seconds at 60 °C. Amplification curves are at figures 9, 10, 11.

RESULTS AND DISCUSSION

The results of the molecular analysis, through the use of real time PCR, support the microscopic analysis of the pollens extracted from some commercialized unifloral honeys.

A PCR operative protocol for the species-specific research of particular pollens has been optimized.

The microscopic observation allowed, in fact, to observe the presence of both pollen granules belonging to the species stated in the honey label and to different species.

Since most of the botanical species used for honey production have an entomophilous pollination, the presence of pollen grains belonging to different species, transported by pollinators, is quite logical. However, in order to be able to declare a honey as unifloral, it is necessary that a certain ratio of concentration be observed between the various pollen grains present.

Real-time PCR results show an amplification both in samples that contained the primers of specific genes of the botanical species that characterize the honey analyzed both in samples that contained primers of genes specific to other botanical species (Figs. 9–11).

In the first case there was a greater amplification and after a lower number of cycles, in the second case the amplification was less evident and delayed.

CONCLUSIONS

The use of microscopic and biomolecular analysis techniques applied to melissopalynology allowed to carry out an evaluation on the pollen composition of some honey commercialized and declared unifloral.

Sample	ng/ µl	260/280	260/230
Eucalypt honey	9.34	2.10	0.70
Sunflower honey	9.76	2,17	0,34
Citrus honey	9,55	1,90	0.65
Miele di Sulla	9.97	2,00	0,80
Acacia honey	8.50	1.60	0,60
Dandelion honey	9.60	1,60	0,52
Linden honey	10,86	1,50	0.60
Asphodel honey	5.26	2,83	0,95

Table 1. Concentration and purity values of the DNA extracted from the analyzed honey.

Species	Pollen percentages
Cardus galactites	5% - 25%
Citrus spp.	> 10%
Castanea sativa	> 90%
Eucalyptus botryoides	> 90%
Rosmarinus officinalis	> 10%
Heliantus annuus	15% - 90%
Hedysarum coronarium	> 50%
Taraxacum officinale	5% - 30%

Table 2. Some percentages of pollen grains belonging to the botanical species characterizing honey according to Ente CRA.

The biomolecular techniques can be used to support classical melissopalynology as a complement to the enhancement of products containing pollens.

This type of investigations are particularly important for checking the correct labeling of honey, in particular when the botanical origin is to be labeled.

According to the Legislative Decree n.179 of 21 May 2004, in order to be declared unifloral, the honeys must have very precise chemical - physical characteristics, such as sugar content, water content and content of substances insoluble in water.

There are also established pollen values belonging to the botanical species that characterizes honey, reported in the characterization charts of each honey, which must be respected.

These values, shown in Table 2, can be widely variable or very rigid, depending on the characteristics of the botanical species.

Sunflower honey, as shown in Table 2, can present quite variable values of Sunflower pollen, ranging between 15% and 90% of the total.

In *Eucalyptus* honey, more than 90% of the pollen grains present in honey must belong to *Eucalyptus* flowers.

Although this type of investigation can still be perfected, a PCR operative protocol for species-specific research of particular pollens has been optimized.

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