determination of pheophytin *a* and pyropheophytin *a* in olive oils.

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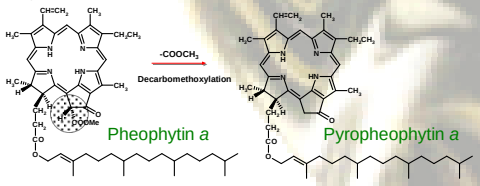
Introduction

Virgin olive oil is obtained from olive fruits (*Olea europaea* L.) by mechanical extraction procedure. During this process, the chlorophylls present in the fruits are transferred to the oil phase due to their lipophyllic character, and at the same time they undergo degradation reactions. The greater accessibility between components during fruit milling and paste beating produces pheophytinization in greater extension and allomerized chlorophylls (13^2-OH-pheophytin and 15^1-OH-lactone pheophytin) in trace amounts. In virgin olive oil obtained from olive cultivars with high chlorophyllase activity, such as Arbequina, de-estherification of phytol takes place, producing chlorophyllides that in acid medium generate pheophorbides (1, 2).

Once produced the virgin olive oil is stored, by a period of time up to one year before marketing. During this period chlorophyll pigments experiences specific changes that imply modification of the pigments profile associated with recently extracted virgin olive oil (3). Quantitatively, there are not losses of pigments, however, qualitatively it is produced a generalized progress of the reactions initiated during the extraction process, pheophytinization and a small increase of allomerization of chlorophylls. In addition, a new type of reaction not taking place during extraction is also detected, namely decarbomethoxylation at C-13^2, that generates a small formation of pyropheophytin *a* (Fig.1). Therefore, the structural pigment transformations will be indicative of the storage time of virgin olive oil (4).

Figure 1



Analysis of chlorophyll pigments in olive oil requires a previous step to eliminate lipid compounds through liquid-liquid extraction (2) or by solid phase extraction (SPE) (5) to obtain a pigment extract suitable for chromatographic analysis. However, by these methodologies it is not feasible the individual quantification of pheophytin *a* and pyropheophytin *a* in oils, due to co-extraction of some proportion of these pigments with the removed lipids.

In the present study we have developed a combine SPE and HPLC, that can be considered as a modification of a previous one (5) in two ways: first assuring a total recovery of pheophytin *a* and pyropheophytin *a* during SPE sample extraction, and secondly, improving selectivity of detection and sensitivity of quantification by using fluorescence detection.

Materials and Methods

Olive oil samples
Stored extra virgin olive oils were used for the present study.

Preparation of standards
Standards for chlorophyll *a*, pheophytin *a* and pyropheophytin *a* were isolated, prepared and purified in our laboratory following general procedures (6, 7).

Solid-phase extraction procedure
Two MiniSped-ed Plus SPE cartridges (500 mg, C18/18%, Applied Separations, USA) fitted in tandem were used for extraction of pigments according to the protocole described in Figure 2. Pigment extracts wereevaporated under nitrogen, and reconstituted in 0.3 ml of acetone.

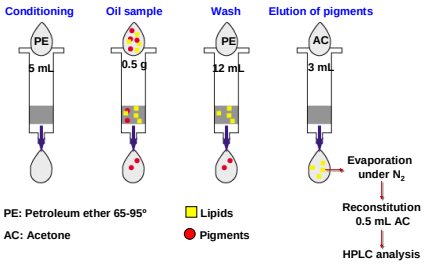
HPLC–DAD–FL instrumentation and conditions
The HPLC system consists of a HP1100 separation module (Hewlett-Packard GmbH, Germany) including a quaternary gradient pump, an autosampler, a thermostatised sample compartment, a Diode Array Detector and a Fluorescence Detector, controled by the HPChem Station System Manager Software (rev A.06.03, Hewlett-Packard Co., USA). For experimental details and conditions see the picture.

Method validation
The method was validated by determination of the following parameters: limit of detection (LOD), linearity range, accuracy, selectivity, and intra-day and inter-day reproducibility. The applicability of the method was demonstrated by the analysis of real samples.

Results and Discussion

Solid-phase extraction (SPE) has been established over recent years as a very effective method for sample pre-treatment and clean-up. It offers various advantages compared to liquid–liquid extraction such as higher efficiency, selectivity and recovery, usage of smaller sample and solvent volumes, ease and convenience in handling, absence of emulsion, less time consumptive, and automation options. Therefore, in order to improve the chromatographic determination a cleaning-up step by means of SPE cartridges was developed (Figure 2). Previous work (5) has demonstrated that lipids can be removed from pigments using reverse phase C18 SPE cartridges, however pyropheophytins could not be quantitatively analysed due to their partial co-extraction with lipid compounds. Therefore SPE of pigments, based on reverse phase was optimized for removing of lipids and retention of pigment. Different solvents (n-hexane, petroleum ether 65-95º, petroleum ether 40-60º, and diethyl ether) and volumes were tested for cartridge conditioning and lipid elution. The best results were obtained by applying the sample to preconditioned cartridge with petroleum ether 65-95º (5 ml). Free-pigmented oil was eluted with 12 ml petroleum ether 65-95º, and subsequently pigments were eluted with acetone (3 ml). More than 96% of oil was efficiently removed, and retention of pigments (pheophytin *a* plus pyropheophytin *a*) were over 97%.

Figure 2. Solid phase extraction (SPE) of chloroplastic pigments from olive oil



To increase the sensitivity and selectivity for the chromatographic analysis a fluorescence detector was connected in series to the diode array detector. Combining a more universal detector like diode array, with a more specific one such a fluorescence detector gives the ability to get more information out of one analysis and directly confirms the identity for pheophytin *a* and pyropheophytin *a* in double. After several attempts the best HPLC results were found using isocratic conditions with acetone-methanol (1:1 v/v) at a flowrate of 2 ml/min. Under these conditions both compounds were separated without interferences within 7 min (3.9 min for pheophytin *a* and 6.7 min for pyropheophytin *a*). This resulted in a method with a total analysis time (elution-, wash- and equilibration period) of 9 min. Figure 3 shows the chromatograms corresponding to the standard pigment mixture (A) and after application of the proposed method to a fresh extracted extra virgin olive oil (B) and to a 12-months stored extra virgin olive oil (C). Detection was simultaneously carried out by UV- visible (410 nm) and in the case of fluorescence this was optimized to Ex: 410 nm and Em: 672 nm. Monitoring of peaks by tandem DAD and FL detectors provided an unequivocally identification of pigments in the oils.

The method was validated and its applicability was demonstrated by the analysis of real olive oil samples. Calibration graphs were linear in the 0.25-14.00 ng/µl and 0.25-19.00 ng/µl concentration ranges (r^2>0.9999) for pyropheophytin *a* and pheophytin *a*, respectively, under both detection conditions. The limit of detection (LOD) defined at a signal-to-noise ratio of 3 was 21.6 ng/g for pyropheophytin *a* and 24.6 ng/g for pheophytin *a* under fluorescence detection, and 148.0 ng/g for both analytes under UV-visible detection. Recoveries of pheophytin *a* and pyropheophytin *a* were over 94%. R.S.D. for intra-day and inter-day determination of pheophytin *a* and pyropheophytin *a* were lower than 3.7% and 8.0%, respectively.

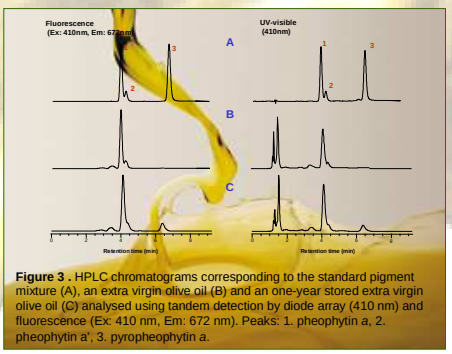


Figure 3 . HPLC chromatograms corresponding to the standard pigment mixture (A), an extra virgin olive oil (B) and an one-year stored extra virgin olive oil (C) analysed using tandem detection by diode array (410 nm) and fluorescence (Ex: 410 nm, Em: 672 nm). Peaks: 1. pheophytin *a*, 2. pheophytin a', 3. pyropheophytin *a*.

The overall process was successfully applied to quantify pheophytin *a* and pyropheophytin *a* in one-year stored extra virgin olive oils. However the absolute content of pheophytin *a* and pyropheophytin *a* may vary from one oil to another depending on the initial chlorophylls content of the fruits, which also depend on many factors such as ripening stage of fruits, seasonal conditions, cultivar, agronomic practices, processing, etc. Therefore the analysis of the relative amount of pheophytin *a* and pyropheophytin *a* (pheophytin *a*/pyropheophytin *a* ratio) promises to be useful as an index for traceability of pigment profile modifications during storage of olive oils.

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