AN INNOVATIVE APPROACH FOR THE PHYTOCHEMICAL ANALYSIS OF BIOACTIVE COMPOUNDS IN *HUMULUS LUPULUS* L.

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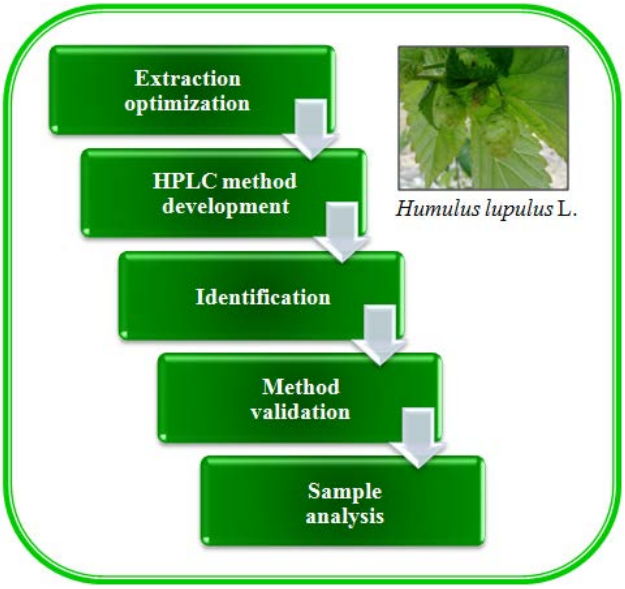
In this work a new tool, comprising a rapid extraction procedure and an efficient analytical method, was developed for the metabolite fingerprinting of hop bioactive compounds. The method was fully validated and successfully applied to commercial cultivars and wild Italian hop genotypes



Un approccio innovativo per l'analisi fitochimica dei composti biologicamente attivi in *Humulus lupulus* L.
Nel presente lavoro è stato sviluppato un metodo nuovo, costituito da una procedura di estrazione rapida e da un metodo analitico efficiente, per il *metabolite fingerprinting* dei composti biologicamente attivi del luppolo. Il metodo è stato completamente validato ed applicato a cultivar commerciali e a genotipi spontanei di luppolo.

Natural products are widely spread throughout the world and herbal supplement companies have expanded considerably into the market. Plant extracts are still today the primary form of health care in the developing countries and are also widely used as supplement or substitute to conventional drugs in the developed ones[1]. Although they are often categorized as "dietary supplements" and not regulated as drugs, these products require a pharmaceutical level of assurance for efficacy and safety in their use[1]. In response to this blending of food and pharmaceutical properties, the term nutraceutical is frequently used.

Plant extracts are composed of a complex mixture of different phytochemicals (plant secondary metabolites). Frequently, these compounds work "synergistically" and cannot be separated into active parts. Consequently, it is necessary to define all the phytochemical constituents of plant extracts to understand the bioactivity and possible adverse effects of active compounds, and to enhance product quality control[2]. *Metabolite profiling*, based on the study of chemically related compounds or secondary metabolites involved in specific biosynthetic pathways, and *metabolite fingerprinting*, based on the full characterization of samples by complete analysis of their secondary metabolites, are highly recommended methodologies in the ambit of natural product analysis[2].

In this ambit, the present work was focused on *Humulus lupulus* L., commonly known as hop, which is a dioecious perennial plant native to the northern hemisphere. The female strobiles (cones) of *Humulus lupulus* L. represent the most interesting part of the plant from a phytochemical point of view. In fact, hop cones are considered an interesting source of natural bioactive compounds[3,4]. In particular, three classes of compounds are significant for their bittering and flavoring properties, and for their health benefits: prenylchalcones (xanthohumol and desmethylxanthohumol), prenylflavanones (isoxanthohumol, 6- and 8-prenylnaringenin) and prenylphloroglucinols, also known as bitter acids or hop acids[3,4] (Fig. 1).

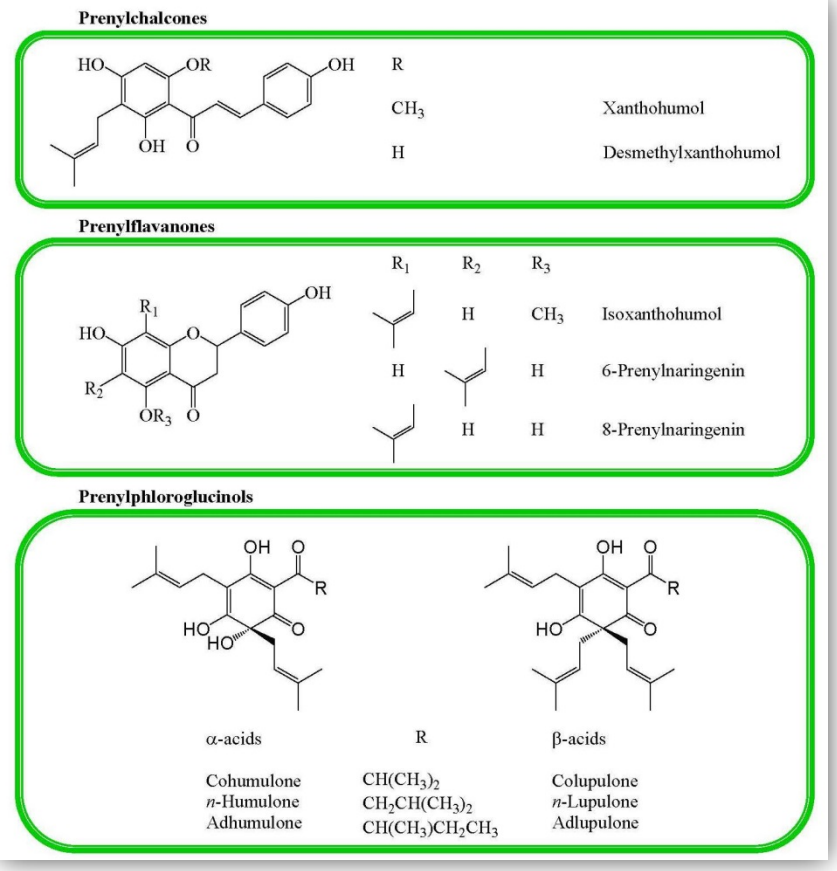


Fig. 1
Chemical structures of prenylchalcones, prenylflavanones and bitter acids of Humulus lupulus L.

Prenylflavanones are almost absent in hop raw plant material and they are known to be generated from the isomerization of prenylchalcones due to thermal treatment or increased pH in aqueous solution[4].
As regards the biological activity, beyond the wide use of hops in the brewing industry to give the typical aroma and bitterness to beer, hop extracts are used in some preparations for their sedative-like activity to treat anxiety and insomnia[3,4] and for their phytoestrogenic properties to treat menopausal complaints[3,4,5]. In particular, the presence of prenylflavonoids in hop extracts has been recognized as the responsible of the phytoestrogenic activity[3,4,5]. As regards the sedative properties of hops, bitter acids seem to be involved in this activity, but their mechanism of action is still unknown[6]. Chemopreventive, antimicrobial, antifungal and stomachic activities have also been described in the literature for hop extracts[3,4].
Several methods have been previously employed to quantify prenylflavonoids and bitter acids in hop plant material, but many of them are focused on single components or are not completely validated[7,8,9,10]. Since the selection of hop varieties with high amount of secondary metabolites can be very useful for their application in phytotherapy, this study was aimed at the complete metabolite fingerprinting of this plant extracts by means of the development of an efficient analytical method, together with a rapid and simple extraction procedure. Furthermore, the complete characterization of the phytochemical profile of hop cones can be used for the selection of interesting varieties for the brewing industry as new flavoring and bittering agents.
In this work, the chromatographic performance of four columns, including two conventional fully-porous and two fused-core, was evaluated. It should be emphasized that the fused-core technology has never been applied before to the analysis of hop extracts. The HPLC analyses were performed using a mobile phase composed of both acidified water and acetonitrile, under a suitable gradient elution. The application of the fused-core column technology allowed a sensitive improvement of the HPLC performance in comparison with that of conventional particulate stationary phases in terms of resolution, sensitivity and analysis time.

As regards the sample preparation, different extraction procedures, including both conventional and modern techniques, were compared in order to obtain a high yield of the target analytes. Dynamic maceration gave the best results in terms of recovery of secondary metabolites and it was chosen for all subsequent analyses.
The metabolite fingerprinting of hop extracts was carried out by means of HPLC-UV/DAD, HPLC-ESI-MS and MS^2, using an ion trap mass analyzer. The complete characterization of prenylflavonoids and bitter acids in hop samples was carried out on the basis of their UV/Vis spectra, together with MS and MS^2 data, which where compared with those of reference standards and with the literature.
The method validation was performed to show compliance with international requirements for analytical techniques for the quality control of pharmaceuticals[11]. Then the validated method was applied to the phytochemical analysis of commercial cultivars and wild Italian hop genotypes. The absence of prenylflavanones demonstrates the reliability of the method, which avoids the isomerization of parent prenylchalcones during the extraction procedure and HPLC analysis. The content of prenylflavonoids and bitter acids of commercial hop samples was in agreement with the literature[12], highlighting that the proposed method works well. Then it was applied to Italian wild samples, showing some varieties with an interesting chemical profile.
In conclusion, the validated method demonstrated to be a reliable and useful tool for the comprehensive multi-component analysis of hop prenylflavonoids and bitter acids. This method can be used in different fields, ranging from routine quality control and standardization of hop extracts to the evaluation of new hop genotypes to be used in phytotherapy or in brewing industry.

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