Contributions to the quality control of two crops of economic importance : hops and yerba mate

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chapter 1

General Introduction

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Man has a vital dependence on plants, using them for all sorts of things including food, clothes, medicines, shelter and fuel and even of course to kill others or even themselves.

The control of the quality of plants however, has only recently become an issue, basically during the second part of the 20th century when the industrialisation and globalisation of trade eliminated any possibility of familiarity with the source of the plants and products made from them.

The increasing popularity of the use of plants, herbs or natural products in general in phytomedicines and a great number of reports of events of diverse severity due to the lack of quality control quickly did away with the widespread prejudice of the inoffensiveness of plants and the health authorities responded with the enforcement of quality control regulations. The EU has been very active through the HMPC (Committee on Herbal and Medicinal Products) created in 2004 as a division of the EMA (European Medical Agency), attempting to harmonise registration, labelling and quality control procedures throughout member countries. At the same time, other countries in the world are looking at this, often translating their decisions to their particular situations and environment.

The use of herbs in food and cosmetics, however, still remains pretty much unregulated. Recently, the FDA through the DSHEA (Dietary Supplements Health and Education Act) in 2004 and the EU through the EFSA (European Food Safety Agency) have enforced similar regulations for the quality control of herbs or herbal ingredients to be used in food.

The regulations that have thus been imposed are exclusively aimed at providing the population with a guarantee of the identity, safety and potency of the herbs or herbal ingredients to be consumed on one hand and the coincidence of the labelling with the content of the product.

The use of product specifications in the manufacture of food items is essential in normal commercial production. A widely used definition of quality in this context is "the collection of features and characteristics of a product or service that confer its ability to satisfy stated or implied needs" (ISO, 1992).

Based on this, a product specification will include the definition of many characteristics, such as nutrient content, energy provided, specific requirements such as

alcohol content in alcoholic beverages and additives. But, even when all these values are standardised, the organoleptic qualities of the product are not necessarily guaranteed, since in most cases attributes such as the taste or aroma of natural products or naturally processed products such as beer, wine, cheese, do not depend on their nutrient content.

So, is a specification included in a regulation enough to qualify a phytoingredient destined to be used as foodstuff? Absolutely not. The quality control of herbal products that are used exclusively as foodstuff directly, or as ingredients for food is generally directed at two major issues that are completely different in their objectives and approaches. The first, most vital issue is the detection of toxic or potentially toxic substances or microorganisms that embody a risk to the consumers' health. The analyses required to detect these do not pose any obvious difficulties and are solved by the application of procedures clearly established by the Health authorities in each country. Naturally, these procedures are not exempt of the flaw common to most quality control methods and that is that they will generally only allow the detection of the substance/s that they are designed for and if unexpected unwanted or toxic substances appear they will most probably not be detected[1].

The other issue that has to be monitored, however, is a lot more complex and consists in the evaluation of flavour and/or the aroma of the ingredient. Actually, this aspect does not only involve the control of these sensory attributes when the food is released by QC (Quality control Dept.), but, more importantly, involves the need to detect stability issues not related to the safety of the product but rather to the maintenance of acceptable flavour and aroma characteristics throughout an economically viable shelf-life period. The most effective approach to solving this very complex problem has been the development of a discipline of sensory analysis based on the use of highly trained people. This is of course, extremely complex, as is evident by the large amount of literature dedicated to discussions on the choice of the right candidates, the interaction between different senses, such as taste and smell, the sensitivity and gender, age, race-conditioned individual differences, the relationship between physiology and psychology, the neutralisation of taints, cultural biases and many more issues (Allen, 2008).

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1 In 1992, in Argentina, 25 people were reported to have died from an intoxication of propolis candy and syrup containing diethylenglycol. This compound was not tested, since it is not part of the formula (naturally) and is not a contaminant of the polyethylenglycol used as an excipient. The laboratory and the owner were exonerated since the tests for this contaminant were not included in the assays requested by health authorities

It has therefore become ever more necessary to develop more objective procedures that could, if not replace, at least provide unbiased information on the taste and flavour of these chemically complex ingredients. The instinctive reaction is to turn to the literature that has been published on the plants themselves. It is of course, massive and can be traced back to the end of the 19th century. The greater part of the first studies were botanical – with a great effort on taxonomical classification. These plants were mostly studied as whole organisms with an accent on the description of individual organs as part of the identification of the species. Later on, as physicists provided more sophisticated analytical tools, the focus was directed at increasingly small structures, zooming from the large macroscopical structures into the smallest details of the composition of anything from the cell wall fibres to the structure of a mitochondrial membrane for example. The detection of all sorts of chemical compounds, their locations, variations, biosynthetic pathways, genes, genetic mapping thus became possible. All this resulted in the publication of dozens of papers per day on plant related research, millions of dollars in subsidies, an amazing feat of human scientific activity.

This would lead us to believe that the chemical composition of plants that are used as food or as a source of medicine, for example, is no secret to us, allowing them to be safely harnessed for our use.

However, if this were the case, standard patterns that guarantee constant taste and aroma, colour, pharmacological activity for example should be relatively easy to achieve. Amazingly this is absolutely untrue and the taste of the plants and the extracts or products made with them –even under very standardised processes– is as formerly explained, only effectively evaluated by the nose and taste buds of specially talented and trained human beings –an analytical "instrument" designed approximately two million years ago.

In the specific case of hops, the fact that it has been used for beer production has led to a gradual improvement of its crops based on the public demand for its organoleptic attributes. This concept is well described by Chadwick, Pauli & Farnsworth (2006) in a paper concerning its medicinal use in which they make an interesting consideration: "Traditionally used herbal medicines have typically been cultivated for hundreds of years or more and distinct genetic lineages have come to exist due to human civilization. In the case of hops, hundreds of named cultivars and many recognized chemotypes exist (Neve, 1991). Hops, as an agricultural crop, in a sense are at a higher state of evolution compared with many other botanicals. The reason for this is due largely to their coveted organoleptic properties that could readily be selected for

in pursuit of the perfect beer. In essence it was bioassay-guided selection, where the bioassay was the beer drinkers' demand for certain flavours. Considering modern bioassay capabilities, similar progress should be seen over the next few decades in the agricultural perfection of other botanicals..."

Clearly the major issue in this case, is that the super specialisation acquired with analytical tools is useless when attempting to establish a taste or aroma pattern, since these are produced generally by the conjunction of numerous chemicals which have not usually been identified since they may be primary or secondary metabolites, active or inactive compounds, and so on.

In this thesis, studies on two plants used in the food industry are reported: hops and mate.

In the first case, the isolation of the major bitter-tasting active principles derived from hops and their extracts –essential ingredients in the beer-brewing process- was undertaken as part of an effort to obtain them in significant amounts and in a highly pure state. These compounds, 3 pairs of isomers of the iso-α-acids derived from the hop bitter acids during the beer brewing process, are not available commercially as pure compounds. Our aim was to obtain them in sufficient amounts to allow the evaluation of the contribution of each individual compound to the bitterness, flavour stability and other characteristics of beer, with the ultimate purpose of developing an objective analytical tool which could allow a relationship between the quantitation of each component and the taste and stability of beer to be established. In order to achieve this, large amounts of high purity compounds were needed.

Our goal was thus to obtain these pure active principles, iso-α-acids, in gram-scale quantities, for which a number of different methods were developed and finally successfully achieved.

In the first part of this thesis, the results obtained with the various methods that were tested or developed for the isolation and production of ultra-pure iso-α-acids will be reported.

The second part deals with the study of aspects of yerba mate, an extremely popular herbal tea made from the leaves of *Ilex paraguariensis*, a plant native to S. America. Much the same as tea or coffee, yerba mate is the product of a long and complicated process of the natural raw material. The quality and taste of this final product depends on multiple factors, starting from the actual leaves that are used, the time of the year in which they are harvested, post-harvest storage, the presence of

adulterants, the amount of stalks included together with the leaves and of course, the processing itself. As in the case of beer, no analytical processes based on the determination of specific compounds seem to solve the quality issues satisfactorily. The presence of adulterants, the influence of the reduction of time-consuming processes on taste, the amount of stalks milled together with leaves or the presence of previous microbial contamination of samples do not show up nor follow any specific pattern in HPLC or GC profiles and have not been able to be pinpointed on any specific compound or groups of compounds.

Thus, in this case a more holistic approach was attempted, using new methods that provide patterns or profiles of the analyte, in order to focus not on individual compounds but on the greatest amount of metabolites as possible. This seemed to be very appropriate considering that in this case, no specific compounds had been related to the characteristic bitter taste of mate, for example. In the case of the detection of possible adulterants, the difficulty lies in the fact that they are basically all Ilex species that have a qualitatively similar chemical composition. The only very different species is *I. paraguariensis* itself, with its high methylxanthine -especially caffeine- content.

In conclusion, the work carried out showed the importance of adapting quality control methods of complex products, such as natural ingredients, to the case in question. For hops, the relevance of the iso-α-acids for the quality attributes of beer had been clearly established. Thus, obtaining the pure compounds was a contribution to the development of methods for controlling the taste and stability of beer. In the case of mate, this approach had proved to be useless when controlling the quality issues described above, since these are clearly related to the raw material or transformations that occur in a number of metabolites or compounds during the processing of *I. paraguariensis* leaves but could not be attributed to specific compounds or groups of compounds.

Part 1

Obtaining pure iso-α-acids from hops (*Humulus lupulus*) in gram-scale amounts

These had to be obtained in gram scale quantities, with ≥ 95% purity. Additionally the impurities could not be toxic for human ingestion nor produce any distortion in taste or aroma.

No methods for obtaining pure iso-α-acids at a relatively large scale had been published at the time of our project or, in fact have been published since, aside from ours. Though these compounds were needed by the industry as reference compounds,

the standards used were and are mixtures of the more stable products of the *trans*-α-isoacid isomers with dicyclohexylamine (DCHA).

The experience in the chemical isolation of iso-α-acids that we counted on at the beginning of our investigation consisted in the interesting method published by Thornton et al. in 1990 that allows the separation of *trans*- and *cis*-iso-α-acids and is used to prepare the *trans*-isohumulone standards used in the beer industry (Thornton et al., 1990,1993). This chemical method consists in the selective precipitation of insoluble complexes / adducts or salts formed between *trans*-iso-α-acids of hops and dicyclohexylamine (DCHA). Thornton reported the reaction between a mixture of iso-α-acids and this reagent, which yields a crystalline product consisting of a mixture of the three *trans*-iso-α-acids (*trans*-isocohumulone, *trans*-isohumulone, *trans*-isoadhumulone) complexes with DCHA. None of the *cis*-iso-α-acids however, precipitate with DCHA remaining thus in the supernatant.

Later on, Maye et al. (1999), modified the method published by Thornton, altering certain conditions used for the formation and recovery of the *trans*-iso-α-acids from DCHA complexes. They reported a yield of 8 g of a *trans*-iso-α-acids mixture starting from 300 ml of 30% aqueous alkaline solution mixture of *cis*- and *trans*-iso-α-acids, i.e., roughly 10% recovery.

This method that provided a way of obtaining the *cis*- and *trans*-iso-α-acids separately, could only be implemented if a few issues were solved. In the first place, DCHA remained as an important contaminant in the *cis*-iso-α-acid supernatant fraction and had to be removed completely. Secondly, it did not allow for the separation of each individual iso-α-acid and thirdly it was necessary to release the *trans*-iso-α-acids from the DCHA complexes, removing all traces of the toxic, foul-smelling and tasting compound.

The application of this method, but starting from individual iso-α-acid isomer pairs provided the basis for our first attempt, as described in Chapter 3.

Another approach for this problem was the use of preparative chromatography. Methods published at that time, included a preparative LC method reported by Verzele and Steenbeke in 1989, who isolated *cis*-iso-cohumulone, *trans*-isocohumulone, *cis*-isohumulone and *trans*-isohumulone from an iso-α-acids extract. However, this method did not allow the isolation of *cis*- and *trans*-iso-adhumulone due to their low concentration in the sample and the poor separation from *cis*-isocohumulone. Later, in a great step forward, P. Hughes was able to develop a method using a preparative HPLC which enabled the separation of individual iso-α-acids with more than 95% purity as determined by HPLC (Hughes, 1996). The latter, though very

successful analytically, could not be up-scaled for larger sample yields without embarking in costly time consuming processes.

However, all previously cited processes entailed several steps, while the light and air-instability of the compounds require a minimum amount of handling to guarantee their integrity.

Based on this previous experience, we considered that pursuing HPLC- based separations would not allow us to obtain all pure compounds at a large scale.

We did decide, however, to use another type of preparative chromatography that had been applied succesfully to the separation of a great number of compounds from natural extracts, and also from hops. Notably, researchers at our department had achieved the separation of the bitter acids from hop extracts using centrifugal-partition chromatography (CPC) with very good results. In 1994, Hermans-Lokkerbol et al. published a CPC method that allowed the separation of the three bitter-α-acids, cohumulone, humulone, and adhumulone, from hop extracts with a 98% purity and a large yield. The method also had the advantage of being relatively simple, cheap and fast, involving a minimum of manipulation.

With this procedure as a starting-point, that is, the pure individual α-acids, we worked on three approaches and a year later we had achieved two things: in the first place, a CPC method which allowed the isolation of the six α-acid isomers, and secondly and perhaps more importantly, a very simple, cheap and up-scalable method based on the selectivity of β-cyclodextrin that allowed the preparation of all six isomers, in a highly pure state and with no traces of toxic impurities.

Part 2

Developing a method for the detection of adulterants in yerba mate.

Ilex paraguariensis, an important S. American crop, is used to make yerba mate, a popular herbal tea. The yerba mate industry is faced with difficult quality control issues, such as how to detect the adulteration of this high caffeine- containing species with other cogeneric native Ilex species.

In this case, after having discussed pending quality issues with the two major manufacturers of yerba mate in Argentina, two topics were undertaken, i.e., the detection of co-generic adulterants in unprocessed Ilex leaf samples and the chemical quantitation of stalks in milled plant material.

All work done on Ilex species had been focussed on detecting the methylxanthine, polyphenolic and saponin content of the leaves, that is, the detection of caffeine and theobromine, present exclusively in *I. paraguariensis* or chlorogenic and

isochlorogenic acids, flavonol glycosides and caffeic acid which are present in both *I. paraguariensis* and co-generic adulterants. As for saponins, though some of those described are specific for each species, the viability of using these diverse saponins as adulteration markers is quite low.

Consequently, HPLC profiles of all species differ mainly quantitatively but not qualitatively, and the possibility of finding a profile unique to *I. paraguariensis*, any deviation from which would denounce the presence of an adulterant, is remote.

We decided to use ^{1}HNMR-metabolomics, a technique used successfully to analyse plant material and extracts, which provides the possibility of applying a holistic approach to the problem. Applying this technique, the wide array of both primary and secondary metabolites detected by ^{1}HNMR spectroscopy in each sample, associated to the reduction of datasets and multivariate analysis of results, allows the detection of discriminating metabolites without the need for reference compounds.

Our first goal was fully achieved, and interestingly a very bioactive compound, arbutin, was found to be present in several of the analysed Ilex species, in some cases in very high amounts (up to 10%). This proved to be very significant as a discriminating metabolite, since there were no previous reports of arbutin in any Ilex species and there is no arbutin in *I. paraguariensis*.

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