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

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summary

This thesis describes the solutions developed for two problems directly related to quality issues of plant-derived raw materials, hops and *Ilex paraguariensis*, used in the manufacture of the massively consumed products, beer and yerba mate. Different types of extracts of the female cones of *Humulus lupulus* (Cannabinaceae) are used in the brewing of beer to confer the typical bitterness primarily, but also due to their foam stabilizing properties. These activities are due to the presence of iso-α-acids formed during the brewing process by the isomerisation of hops bitter α-acids, humulone, cohumulone and adhumulone, which yield 3 pairs of stereoisomers, *trans/cis*-isohumulone, -isocohumulone, -isoadhumulone, respectively.

Although it is clear that these compounds are directly responsible for the bitterness of beer, the extent of their individual contribution was not absolutely clear, and the only way to evaluate this was to have a sufficient amount of the individual highly pure isomers. These are not commercially available. Further, the only available iso-α-acid reference standards are mixtures of dicyclohexylamine (DCHA) salts of the *trans*-iso-α-acids, clearly not suited for sensory taste panels. We set out, thus, to find a way to produce these key compounds for sensory quality testing of beer.

The methods were based on the combination of chromatographic separations using a powerful preparative chromatography technique, centrifugal partition chromatography (CPC) and selective *trans* /*cis* precipitation based methods. Our first efforts consisted in the CPC separation of the three bitter-α-acids, humulone, cohumulone and adhumulone using a method developed by Hermans-Lokkerbol *et al.* (1997), their subsequent alkali-magnesium catalysed isomerisation using a slightly modified version of Koller's method (1968) and the selective precipitation of *trans*-iso-α-acid using DCHA (Thornton *et al.*, 1993). This allowed the separation of the all six isomers, but the yield was extremely low, due to low recovery rates of the process of regenerating the *trans*-isomers from the DCHA salt and the removal of the most minimal trace of the toxic, disagreeable DCHA. On the other hand, this method allowed us to produce individual DCHA-*trans*-iso-α-acids that are not available as such commercially. The pure individual *cis*-counterparts could be made available also as methanolic solutions to be used as reference compounds. (Chapter 3)

The second strategy depended more strongly on CPC separations. Using an isomerised hop extract or an CO_2 hop extract that we isomerised, we separated the *trans*/*cis* isomers to obtain all *trans*- or all-*cis* mixtures using DCHA. These samples were then

submitted to CPC. The best separation was achieved with a pH-zone refining method, using a ternary system consisting of n-hexane: methanol: water (10:5:5) + 2% TFA in the upper organic phase as a stationary phase and the lower aqueous phase+ 0.05% ammonia as a mobile phase. After 1.5 hours, the amount of ammonia was increased to 0.1%; the flow rate was 2.0 ml/min, revolution speed was fixed at 900 rpm and the eluate was monitored at 270 nm. In this case the DCHA was practically all removed during the CPC separation, and the pure isomers were obtained. The application of the same method to an isomerised hop extract to separate all 6 isomers in one same chromatographic run allowed us to obtain 3 of the 6 isomers in a pure state: *cis*-isohumulone, *cis*-isoadhumulone and *trans*-isohumulone. *Trans*-isoadhumulone which together with *cis*-isoadhumulone is a minor component, was not able to be collected in any significant amount and *trans*-isohumulone eluted in a low proportion during one hour approximately, contaminating and decreasing the purity of *trans*-isocohumulone (from which it was also partly separated) and *cis*-isocohumulone. (Chapter 4).

The method that proved to be the best was based on the CPC separation of a CO_2 hop extract, followed by the alkali-catalysed isomerisation of the α-acids and complexation with β-cyclodextrin that yielded only *trans*-iso-α-acid/β-cyclodextrin inclusion complexes. The *cis*-iso-α-acid that remained in solution could be recovered by extraction with a non-polar solvent and kept in an ethanolic solution. The white powdered β-cyclodextrin complex with *trans*-iso-α-acids proved to be very stable. Furthermore, when this method was applied to an isomerised hop extract, it was possible to obtain an all-*trans*-iso-α-acid extract. The complexation was achieved using a β-CD solution prepared by dissolving 1,8 g (equivalent to 1,58 x 10^{-3} mols) of β-CD in 18 ml ethanol: water (1:2, v/v) and heating to 50°C. The samples were prepared by dissolving approximately 0.5 g iso-α-acids (equivalent to 1,57 x 10^{-3} mols, considering an average MW=326da) in 6.5 ml ethanol. This solution was added dropwise to 18 ml of the β-CD solution, while continually stirring at 50 °C for 30 min. The mixture was stored at 4 °C for 3 days in absence of light after which an off-white crystalline solid corresponding to the *trans*-iso-α-acids complex precipitated, leaving a transparent colourless supernatant (Chapter 5).

The second part of the thesis deals with *Ilex paraguariensis*, a plant used to make the extremely popular S. American tea, Yerba mate. Its local economic relevance and the extent of its rapidly increasing use as a herbal tea are described in Chapter 6; the fact that the industrialisation of yerba mate is relatively recent explains the need for the development of better quality parameters that could help to improve the processes so that the final product is safer and has more consistent sensory attributes. There is

special interest in promoting yerba mate to the category of a functional food, so that it is important to achieve consistent standards.

In Chapter 7, a review of its chemical composition and bioactivities are discussed. The major secondary metabolites in *Ilex paraguariensis* are xanthines, mainly caffeine and lower amounts of theobromine, caffeoylquinic acid derivates (mainly neo-chlorogenic and chlorogenic acid) and saponins. Most investigations of *Ilex* species date back to the '80s, but even though relatively recent, results are remarkably inconsistent. Variations in the xanthine content, that include some controversy on the presence of theophylline even, and polyphenolic content are very usually difficult to explain if not through the shortcomings of older HPLC instrumentation for example, in which compounds were identified solely by their retention times. New methods like LC/MS and ^{1}HNMR are beginning to clarify some of these issues, so that all previous work is worth reviewing in a critical light. In the case of the alleged bioactivities, it is clear that further work should be done or at least that available results should be reinterpreted more objectively and with more rigorous standards, since a lot of the activities are often reported in doses or levels which are not considered to be active (antimicrobial activities, for example). All these issues are important considering that Yerba mate and its derivatives are used medicinally, and as such, its monograph is included in the Argentine and Brazilian pharmacopoeias as well as the Pharmacopee Francaise and the DAB. There is also a European Community monograph under the name of *Mate folium.*

As discussed in Chapter 7, all *Ilex* species have a similar qualitative composition, and while *I. paraguariensis* is the odd-man out with its high xanthine content, all other phytochemicals are very similar. In Chapter 8 we describe a possible solution to one of the many issues affecting the quality of this herbal infusion, i.e., the implementation of a method to detect the adulteration of yerba mate leaves with cogeneric *Ilex* species. Using the typical quality control methods based on the detection of certain compounds or markers had proved to be useless. Instead we applied a holistic approach, implementing a method that would allow the detection of as many metabolites as possible. An ^{1}HNMR-based metabolomics method applied to the analysis of 11 *Ilex* species, *I.paraguariensis, I. dumosa, I. argentina, I. taubertiana, I.pseudobuxus, I. microdonta, I. theezans, I. brasiliensis, I. dumosa* var *dumosa, I. integerrima,* allowed us to discriminate the 11 species. The major metabolites involved in the discrimination were polyphenols and saponins. At the same time, when obtaining the ^{1}HNMR data, we detected the presence of arbutin in amounts ranging between 2.23-10.60 10% (dw). Arbutin, a glycoside that is hydrolysed before absorption in the gastrointestinal tract to give hydroquinone, is a strong urinary antiseptic among other activities and is the

active principle of *Uva ursi*, for example, which contains similar amounts to *I.brasiliensis*. This had never been detected in any *Ilex* species.

These results were confirmed by HPLC using a method consisting of a C18 column (Inertsil ODS-3 (4.6 x 250 mm-5μ)) isocratic elution for 4 minutes with AcOH/H_2O 1%: AcOH/MeOH 1% (90:10), and a linear gradient to 30:70 in 26 minutes. Arbutin was detected at 282nm, and at a flow rate of 1.0 ml/min, eluted at 4.83 ± 0.5min. The fully validated method and its results are described in Chapter 9.