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Metabolic classification of South American *Ilex* species by NMR-based metabolomics

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ABSTRACT

The genus *Ilex* to which mate (*Ilex paraguariensis*) belongs, consists of more than 500 species. A wide range of metabolites including saponins and phenylpropanoids has been reported from *Ilex* species. However, despite the previous works on the *Ilex* metabolites, the metabolic similarities between species which can be used for chemotaxonomy of the species are not clear yet. In this study, nuclear magnetic resonance (NMR) spectroscopy-based metabolomics was applied to the classification of 11 South American *Ilex* species, namely, *Ilex argentina*, *Ilex brasiliensis*, *Ilex brevicuspis*, *Ilex dumosa* var. *dumosa*, *I. dumosa* var. *guaranina*, *Ilex integerrima*, *Ilex microdonta*, *I. paraguariensis* var. *paraguariensis*, *Ilex pseudobuxus*, *Ilex taubertiana*, and *Ilex theezans*. ^{1}H NMR combined with principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA) and hierarchical cluster analysis (HCA) showed a clear separation between species and resulted in four groups based on metabolomic similarities. The signal congestion of ^{1}H NMR spectra was overcome by the implementation of two-dimensional (2D)-*J*-resolved and heteronuclear single quantum coherence (HSQC). From the results obtained by 1D- and 2D-NMR-based metabolomics it was concluded that species included in group A (*I. paraguariensis*) were metabolically characterized by a higher amount of xanthines, and phenolics including phenylpropanoids and flavonoids; group B (*I. dumosa* var. *dumosa* and *I. dumosa* var. *guaranina*) with oleanane type saponins; group C (*I. brasiliensis*, *I. integerrima*, *I. pseudobuxus* and *I. theezans*) with arbutin and dicaffeoylquinic acids, and group D (*I. argentina*, *I. brevicuspis*, *I. microdonta* and *I. taubertiana*) with the highest level of ursane-type saponins. Clear metabolomic discrimination of *Ilex* species and varieties in this study makes the chemotaxonomic classification of *Ilex* species possible.

1. Introduction

The genus *Ilex* comprises more than 500 species of dioecious trees and shrubs distributed throughout temperate and tropical regions of the world (Galle, 1997). It is one of the main genera of the Aquifoliaceae family together with the monospecific genus *Nemopanthus* of eastern North America (Noud et al., 2000). Many species of *Ilex* are ornamental (holly plant). However, one of these species, *Ilex paraguariensis*, has been used since pre-Columbian times and later grown as a domesticated crop in the northeastern region of Argentina and South-east of Brazil where its leaves are processed to produce yerba mate (Maria et al., 1997). Mate is massively consumed in this region either as a decoction or a type of concentrated infusion, much the same as tea or coffee in other parts of the world. Similarly to these, it contains significant amounts of the stimulant xanthine, caffeine.

A number of therapeutic applications have been claimed for mate infusions, such as, choleretic (Gorzalczany et al., 2001), anti-inflammatory (Peluso et al., 1995) anti-ageing; anti-obesity (Anderson and Fogh, 2001), anti-oxidant (Filip et al., 2000), diuretic (Gonzalez et al., 1993), anti-thrombotic (Gugliucci and Menini, 2002), and endothelium-dependent vasorelaxing activity (Mucillo-Baisch et al., 1998). It has also recently begun to be used to reduce fatigue and as an appetite suppressant (Cardozo et al., 2007).

Among the several hundreds of existing *Ilex* species, a few of them such as *Ilex brevicuspis* and *Ilex theezans* grow in the same habitat of *I. paraguariensis*. Others, as in the case of *Ilex argentina* Lillo known as 'árbol de la yerba' or 'palo de yerba', are native to the northwestern Argentine region. All of these are considered to be potential substitutes or adulterants of *I. paraguariensis* (Giberti, 1989) in the commercial production of yerba mate. The possibility of detecting the presence of these adulterants is therefore an

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important issue in the quality control of yerba mate for which there is no satisfactory solution to date.

The chemical composition of *Ilex* species growing in this region has been extensively studied. A wide range of primary and secondary metabolites have been described, among which phenolics, including flavonoids and phenylpropanoids have been reported in all species (Filip et al., 2001). Phenylpropanoids and caffeic acid analogues have been isolated from *I. paraguariensis* (Clifford and Ramirez-Martinez, 1990). Apart from these, as mentioned above, the xanthine caffeine has also been detected in *I. paraguariensis* (Filip et al., 1998, 2001; Athayde et al., 2000; Cardozo et al., 2007). These authors also reported the presence of lower amounts of another xanthine – theobromine – while the presence of theophylline, reported in very small quantities in some papers (Mazzafera, 1994), is a matter of controversy, as several researchers were unable to detect this substance (Baltassat et al., 1984; Clifford and Ramirez-Martinez, 1990; Ashihara, 1993; Filip et al., 1998). The xanthine content of *I. paraguariensis* preparations such as decoctions has also been studied together with that of other South American *Ilex* species. Among these, *I. paraguariensis* exhibited the highest levels of caffeine and theobromine, while no xanthines were detected in *I. brevicuspis*. *I. theezans* was reported to contain traces of caffeine and theophylline and *I. argentina* traces of theobromine (Filip et al., 1998). Apart from these aromatic compounds, pentacyclic triterpenoids such as α- and β-amyrin, and ursolic and oleanolic acids have also been found in *Ilex* species (Niemann and

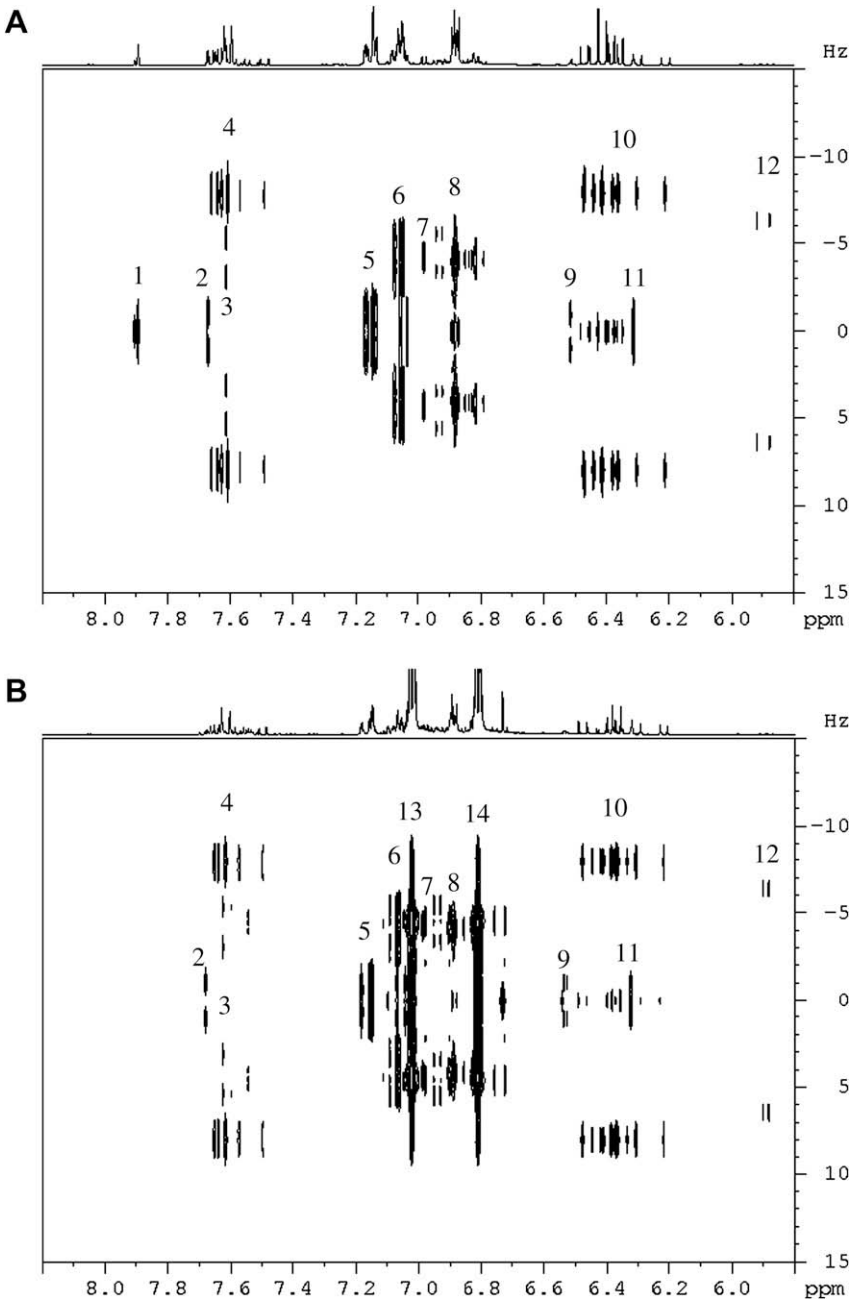


Fig. 1. 2D ^{1}H–^{1}H *J*-resolved spectrum of *I. paraguariensis* (number of voucher specimen: 28) (A) and *I. theezans* (number of voucher specimen: 16) (B) extracts in the range of δ 5.8–8.2. (1) H-8 of caffeine, (2) H-2′ of rutin, (3) H-6′ of rutin, (4) H-7 of *trans*-phenylpropanoids, (5) H-2 of *trans*-phenylpropanoids, (6) H-6 of *trans*-phenylpropanoids, (7) H-5′ of rutin, (8) H-5 of *trans*-phenylpropanoids, (9) H-8 of rutin, (10) H-8 of *trans*-phenylpropanoids, (11) H-6 of rutin, (12) H-8 of *cis*-phenylpropanoid, (13) H-1′ and H-6′ of arbutin, (14) H-3′ and H-5′ of arbutin.

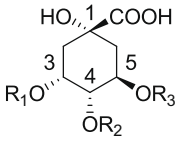
Baas, 1985). A number of papers have been published reporting the saponin content of South American *Ilex* species (Schenkel et al., 1997), or more specifically of characteristic saponins, such as matesaponins in *I. paraguariensis*, (Gosmann et al., 1989, 1995; Kraemer et al., 1996) and in *Ilex dumosa* (Heinzmann and Schenkel, 1995), or brevicuspisaponins from *I. brevicuspis* (Taketa et al., 2000) among others.

In our previous reports, a possible chemical marker of species of the *Ilex* species, namely *I. argentina*, *Ilex brasiliensis*, *I. brevicuspis*, *Ilex integerrima*, *Ilex microdonta*, *Ilex pseudobuxus*, *Ilex taubertiana*, and *I. theezans* was identified. All of these species were found to contain arbutin as a major metabolite, while it is not present in *I. paraguariensis*. Among the 11 *Ilex* species and varieties that were analyzed, *I. brasiliensis*, *I. pseudobuxus* and *I. theezans* showed exceptionally high amounts of arbutin (ca. 100 mg/g) as compared to other species (Choi et al., 2005). Although there are many *Ilex* species present in nature, the classification of which might be intricate in terms of taxonomy, there are only few reports on the systematic metabolic characterization of *Ilex* species. Moreover, an explicit characterization could be employed in the separation of *I. paraguariensis*, unique among *Ilex* plants for its human consumption, and its adulterants.

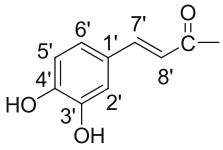
Metabolomics is the systematic identification and quantification of all metabolites in a given organism or biological sample. Naturally, the analytical platform for the acquisition of data on metabolite content is critical for the success of the studies. Among the several candidates, nuclear magnetic resonance (NMR) spectroscopy has proved to allow the detection of a wide range of metabolites with high robustness and requiring only a very simple sample preparation. Even one of the major drawbacks for NMR application, low sensitivity, can eventually be in part overcome by recently developed cryo (cold)-probe technology. The enhanced resolution and sensitivity provided by NMR spectroscopy along with powerful chemometric tools, allows metabolomics to be applied to diverse fields of plant science.

Metabolomic profiling through ^{1}H NMR spectroscopy based on classification and characterization has been profusely used for plants and plant-derived preparations. Some good examples of this are the differentiation of *Cannabis sativa* cultivars (Choi et al., 2004), the classification of *Ephedra* species and commercial *Ephedra* herbs (Kim et al., 2005), the discrimination of commercial feverfew preparations (Bailey et al., 2002) and commercial samples of catuaba (Daolio et al., 2008). In a previous article we reported the metabolomic analysis of 11 *Ilex* species including two varieties, using an NMR spectroscopic method coupled to principal component analysis (PCA) and soft independent modeling class analogy (SIMCA) (Choi et al., 2005). On the basis of this data, the *Ilex* species employed in the study were well characterized metabolically. However, the application of NMR posed some problems such as the tedious time-consuming sample preparation step and overlapping of ^{1}H NMR signals that hinder robust metabolite identification. Additionally, the use of PCA and SIMCA limited the detection of metabolic resemblance between two species. As a result, the classification of all analyzed samples was not achieved in previous studies.

In order to avoid these problems, two changes were made. In the first place, deuterated NMR solvents were used to extract the *Ilex* samples thereby reducing sample preparation time. Secondly, two-dimensional (2D)-^{1}H–^{1}H *J*-resolved and heteronuclear single quantum coherence (HSQC) spectroscopy were applied with the purpose of identifying as many metabolites as possible from the overlapped ^{1}H NMR signals. In addition to these analytical improvements, hierarchical cluster analysis as a successive chemometric approach was applied to the generated principal

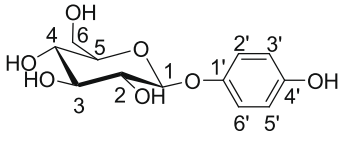


quinic acid moiety

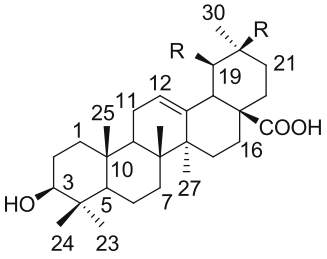


caffeoyl moiety

R_1 = caffeoyl, R_2 = R_3 = H, 3-*O*-caffeoyl quinic acid
R_1 = R_3 = H, R_2 = caffeoyl, 4-*O*-caffeoyl quinic acid
R_1 = R_2 = H, R_3 = caffeoyl, 5-*O*-caffeoyl quinic acid (chlorogenic acid)
R_1 = R_2 = caffeoyl, R_2 = R_3 = H, 3,4-*O*-dicaffeoyl quinic acid
R_1 = R_3 = caffeoyl, R_2 = H, 3,5-*O*-dicaffeoyl quinic acid
R_1 = H, R_2 = R_3 = caffeoyl, 4,5-*O*-dicaffeoyl quinic acid



arbutin



R_1 = H, R_2 =CH_3, oleanolic acid
R_1 = CH_3, R_2 =H, ursolic acid

Fig. 2. Chemical structures of phenylpropanoids, arbutin and triterpenoidal moieties of saponins identified by NMR spectra in *Ilex* species.

components (PCs) and partial least square (PLS) components obtained from PCA and PLS-discriminant analysis thus increasing the reliability of the metabolic characterization and contributing to the determination of the degree of metabolic closeness between species.

2. Results and discussion

2.1. Identification of metabolites in the extracts using 2D-NMR

In our previous report, a two-phase extraction method using chloroform–MeOH–water followed by evaporation and reconstitution with deuterium solvents was used for *Ilex* samples (Choi et al., 2005). However, this method involved a long sample preparation time, apart from the possibility of the loss of metabolites or thermally induced degradation or alterations, e.g., isomerization of phenylpropanoids. Thus, in this study we directly used deuterated solvents for extraction – a CH_3OH-d_4 and KH_2PO_4 buffer in D_2O (1:1) – simplifying the tedious sample preparation step. These extracts allowed the detection of a wide range of metabolites. For example, using the previous two-phase extraction method, caffeine and chlorogenic acid were separately extracted in the chloroform and water fraction respectively, while in this case both metabolites were well detected in the single extract.

In order to deal with the signal congestion, 2D-NMR spectra were employed. Although ^{1}H NMR has been considered a promising analytical tool for metabolomics research, a number of problems have yet to be solved. One of the most troublesome is signal overlapping. In this case, signal congestion was solved applying diverse 2D-NMR spectra to the deconvolution of metabolites in the *Ilex* extracts. Among the spectra employed,

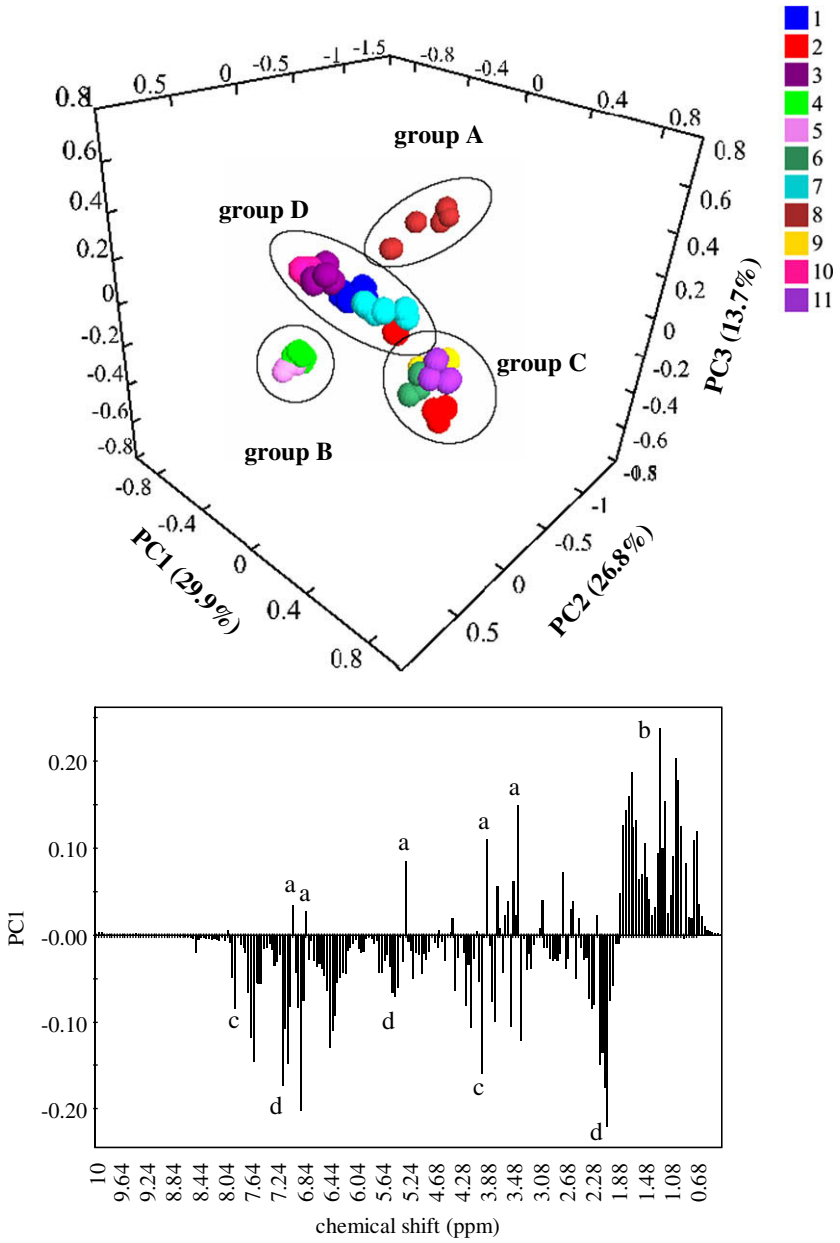


Fig. 3. Score plot of principal component analysis (PCA) results obtained from ^{1}H NMR using PC1, PC2 and PC3 (A) and loading plot of PC1 (B). Group A: *I. paraguariensis* var. *paraguariensis* (8). Group B: *I. dumosa* var. *dumosa* (4) and *I. dumosa* var. *guaranina* (5). Group C: *I. brasiliensis* (2), *I. integerrima* (6), *I. pseudobuxus* (9) and *I. theezans* (11). Group D: *I. argentina* (1), *I. brevicuspis* (3), *I. microdonta* (7) and *I. taubertiana* (10). (a) Arbutin signals, (b) methyl signals of saponin, (c) caffeine signals, (d) caffeoylquinic acids and rutin signals.

2D-^{1}H–^{1}H *J*-resolved spectrum has previously showed promising results in terms of time efficiency and signal robustness in diverse applications of metabolomics (Viant, 2003; Choi et al., 2006). In this case, many overlapped ^{1}H NMR resonances were clearly resolved alongside to F_1-axis of coupling constants in *J*-resolved spectrum (Fig. 1). In the aromatic region, the resonances of various phenylpropanoids were well separated from xanthines and flavonoids. In the past, other research groups have reported that the major phenolic compounds of *Ilex* species are chlorogenic acid, 3,4-, 3,5- and 4,5-*O*-dicaffeoylquinic acid as well as rutin (Clifford and Ramirez-Martinez, 1990; Ricco et al., 1991; Filip et al., 2001). All of these major phenolics were clearly detected in the 2D-*J*-resolved spectrum with high resolution, characterized particularly by the signal corresponding to H-8′ or H-8″ of phenylpropanoids in the range of δ 6.1–6.5 which shows the presence of a variety of phenylpropanoids. The ^{1}H resonances of phenylpropanoids, H-8′ or H-8″ resonances (*d*, *J* = 16.0 Hz) of phenylpropanoids are clearly influenced by substitution and can thus be used for identification of individual phenylpropanoids. Based on the spectra of isolated compounds, our in-house database and reference literature (Choi et al., 2006; Pauli et al., 1998), chlorogenic acid (5-*O*-caffeyoyl quinic acid) (H-8′ at δ 6.36) as well as three dicaffeoylquinic acids such as 3,4-*O*- (H-8′ and H-8″ at δ 6.45 and δ 6.37), 3,5-*O*- (H-8′ and H-8″ at δ 6.47 and δ 6.39) and 4,5-*O*-dicaffeoylquinic acid (H-8′ and H-8″ at δ 6.30 and δ 6.21) were identified in *I. paraguariensis* and *I. theezans* extracts (Fig. 2). In other species, instead of dicaffeoylquinic acids, 3-*O*- and 4-*O*-caffeoylquinic acids were detected as major phenylpropanoids as well as chlorogenic acid. In addition to these mono- and dicaffeyol quinic acids, a major flavonoid, rutin was clearly detected in the aromatic region of *Ilex* extract due to its resonances at δ 6.31 (H-6, *d*, *J* = 2.0 Hz), δ 6.53 (H-8, *d*, *J* = 2.0 Hz), δ 6.98 (H-5′, *d*, *J* = 8.5 Hz), δ 7.62 (H-6′, *dd*, *J* = 8.5 Hz, 2.1 Hz) and δ 7.69 (H-2′, *d*, *J* = 2.1 Hz) as well as anomeric protons at δ 5.01 (glucosyl H-1″, *d*, *J* = 7.7 Hz) and δ 4.54 (rhamnosyl H-1‴, *d*, *J* = 1.3 Hz). Characteristic arbutin ^{1}H NMR resonances were found at δ 7.03 (*d*, *J* = 9.0 Hz) and δ 6.81 (*d*, *J* = 9.0 Hz) in several *Ilex* species such as *I. argentina*, *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. pseudobuxus*, *I. taubertiana* and *I. theezans* as described in our previous report (Choi et al., 2005). The complex mixture of *Ilex* phenylpropanoids, flavonoids and arbutin are well detected with high resolution in a single run of 2D-*J*-resolved spectrum without the need of a chromatographic separation. In the case of xanthines, only *I. paraguariensis* showed detectable amounts of the H-8 of caffeine and theobromine although there are some reports on the presence of minor amounts of xanthines in other species (Filip et al., 1998). This 2D-*J*-resolved spectrum can thus be used for the metabolic fingerprinting of *Ilex* species.

In our previous report, arbutin was described as a chemical marker in *I. argentina*, *I. brasiliensis*, *I. brevicuspis*, *I. integerrima*, *I. microdonta*, *I. pseudobuxus*, *I. taubertiana*, and *I. theezans*. However, in 2004, Andrade et al. reported on the identification of arbutin-2-sulfonyl from *I. theezans* leaves (Andrade et al., 2004). To confirm the presence of this form of arbutin, diverse 2D-NMR spectra such as COSY, HSQC and HMBC were used for the detection. However, this sulfonyl was not detected in the extract of *I. theezans*. H-2 of the sulfonyl gives shifts from δ 3.49 to δ 4.30 (Andrade et al., 2004). In the COSY spectrum of the *I. theezans* extract, H-1 correlates only with δ 3.49. Additionally, the ^{13}C chemical shift of C-2 of arbutin-2-sulfonyl would be shifted downfield from δ 75.9 to δ 82.0. In this study, however, the HMBC spectrum of *I. theezans* extract showed the correlation of H-1 only with δ 75.9 (C-2 of arbutin) and δ 152.2 (C-1′ of arbutin). Based on these results, it was concluded that major form of arbutin is not as a sulfonyl but rather as a free form.

2.2. Metabolomic classification of Ilex species using PCA, HCA and PLS-DA

^{1}H NMR data was reduced by principal component analysis in order to obtain the maximum variation between the samples. A 33-component model explained 99.7% of the variance, with the first three components explaining 70.4%. Using PC1, PC2 and PC3, the 11 *Ilex* species and varieties employed in this study were found to be clustered into four groups (Fig. 3A). *I. paraguariensis* (8, group A) is clearly separated from other samples. The loading plot of PC1 (Fig. 3B) indicated that the amount of caffeoylquinic acids, rutin and caffeine was higher in *I. paraguariensis*, while the level of saponins and arbutin were found to be relatively lower than in the other species. The other groups were formed by two varieties of *I. dumosa* (4 and 5, group B) in (Fig. 3A); *I. brasiliensis* (2),

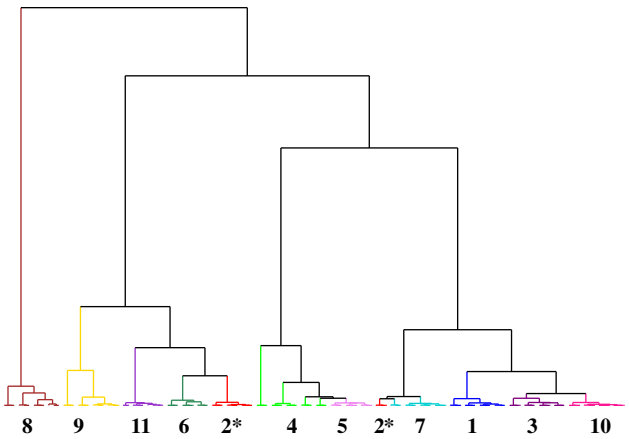


Fig. 4. Dendrogram of HCA results based on 33 PCs obtained from PCA. (1) *I. argentina*, (2) *I. brasiliensis*, (3) *I. brevicuspis*, (4) *I. dumosa* var. *dumosa*, (5) *I. dumosa* var. *guaranina*, (6) *I. integerrima*, (7) *I. microdonta*, (8) *I. paraguariensis* var. *paraguariensis*, (9) *I. pseudobuxus*, (10) *I. taubertiana*, (11) *I. theezans*. ∗: not classified.

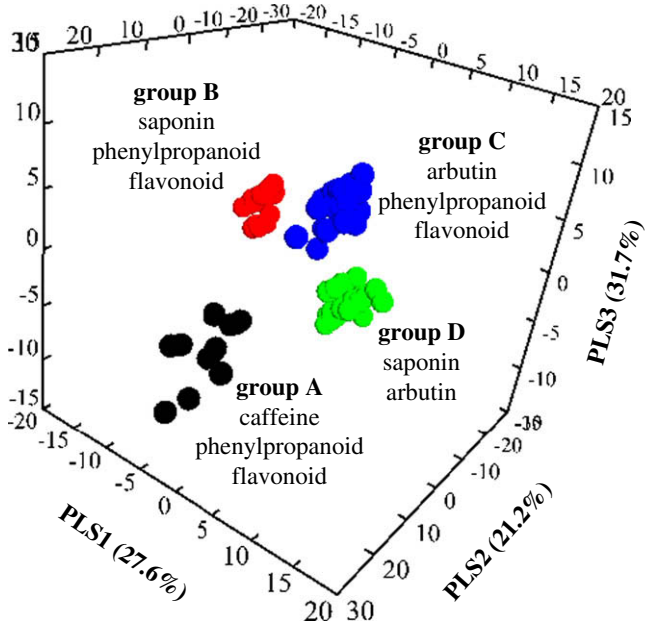


Fig. 5. Score plot of partial least square-discriminant analysis (PLS-DA) results obtained from ^{1}H NMR using PLS1, PLS2 and PLS3. Group A: *I. paraguariensis* var. *paraguariensis* (8). Group B: *I. dumosa* var. *dumosa* (4) and *I. dumosa* var. *guaranina* (5). Group C: *I. integerrima* (6), *I. pseudobuxus* (9) and *I. theezans* (11). Group D: *I. argentina* (1), *I. brevicuspis* (3), *I. microdonta* (7) and *I. taubertiana* (10).

I. integerrima (6), *I. pseudobuxus* (9) and *I. theezans* (11) in another group (group C) and lastly group D consisting of *I. argentina* (1), *I. brevicuspis* (3), *I. microdonta* (7) and *I. taubertiana* (10). However, the location of two of the species, *I. brasiliensis* (2) and *I. pseudobuxus* (9) was not clear, probably belonging to group C or D.

Although the PCA score plot provides some clues for grouping, the available PCs are limited because only three PCs can be graphically shown. Also, the score plot does not provide any information on the closeness between groups. Applying hierarchical cluster analysis (HCA) can help to obtain further information on these aspects. For the HCA, 33 PCs reduced from the original ^{1}H NMR signals were used. As shown in Fig. 4 four groups were obtained from HCA. The grouping in HCA is the similar as that of PCA. *I. paraguariensis* is clearly separated from other species. Among the other *Ilex* species employed in this study, the metabolome of *I. integerrima* (6), *I. pseudobuxus* (9) and *I. theezans* (11) were found to be the most similar to that of *I. paraguariensis*, while the furthest species was found to be *I. taubertiana* (10) in terms of their metabolomes. In the case of *I. brasiliensis*, the classification based on metabolomic analysis is not clear because there is a rather large metabolomic variation between the samples as compared to the other species.

The *Ilex* species used in this study were grown in the same conditions after collecting the seeds from diverse places in South America. Thus, it is expected that the metabolomic differences observed could not be attributed to external factors such as climate, soil condition or water stress but rather to the inherent character of each species such as their genetic composition.

These conclusions coincide with the chemotaxonomical results obtained by the application of molecular analysis with amplified fragment length polymorphism to *Ilex* species (Gottleib et al., 2005). For example, their results showed a very close relationship between *I. brasiliensis*, *I. integerrima* and *I. theezans*, which was consistently reflected in our metabolomic analysis (Fig. 4).

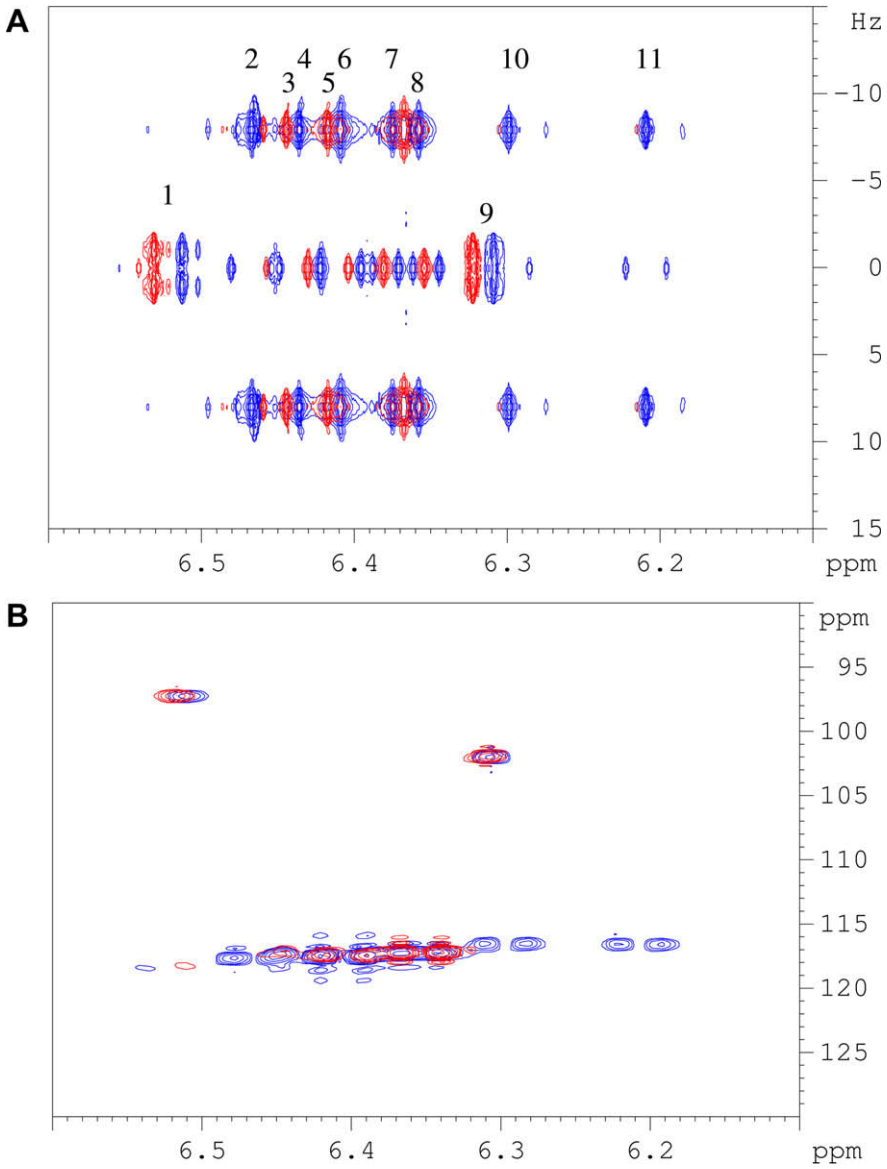


Fig. 6. 2D-*J*-resolved (A) and HSQC spectra (B) of *I. paraguariensis* (number of voucher specimen: 28) and *I. dumosa* var. *dumosa* (number of voucher specimen: 7) in aromatic range (δ 6.1–6.6 for ^{1}H and δ 90–130 for ^{13}C). Blue: *I. paraguariensis*, red: *I. dumosa* var. *dumosa*. (1) H-8 of quercetin glycosides, (2) H-8′: 3,5-*O*-dicaffeoylquinic acid, (3) H-8′: 4-*O*-caffeoylquinic acid, (4) H-8′: 3,4-*O*-dicaffeoylquinic acid, (5) H-8′: 3-*O*-caffeoylquinic acid, (6) H-8″: 3,5-*O*-dicaffeoylquinic acid, (7) H-8″: 3,4-*O*-dicaffeoylquinic acid, (8) H-8′: 5-*O*-caffeoylquinic (chlorogenic acid), (9) H-6 of quercetin glycosides, (10) H-8′: 4,5-*O*-dicaffeoylquinic acid, (11) H-8″: 4,5-*O*-dicaffeoylquinic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

From the metabolomic similarity obtained from PCA and HCA, these 11 *Ilex* species and varieties can thus be divided into four groups. However, the PCA grouping can be only obtained from arbitrary visual investigation because PCA aims at the separation of each individual sample by maximum variation. Consequently, a supervised method requiring additional Y-datasets is needed to confirm the grouping of *Ilex* species. With the exception of *I. brasiliensis* which showed a large variation in PCA, four groups were analyzed by PLS-DA. Fig. 5 shows that quite a clear metabolomic discrimination of each group was achieved. Also, PLS-DA provides a correlation between metabolites and groups, so the detection of the metabolites which act as chemical markers of each *Ilex* group can be expected. The loading plot shows the correlation between bucketed chemical shifts of ^{1}H NMR spectra and the group. Of the correlated ^{1}H NMR signals, significant values were selected from the variable importance for projection (VIP) of each signal, which is calculated by adding the squares of PLS-weights, weighted by the amount of Y-explained in each model component. All of the major known *Ilex* metabolites such as phenylpropanoids, flavonoids, saponins and arbutin are discriminating metabolites for each group but the combination of differentiating metabolites varies depending on the species. For example, *I. paraguariensis* was found to contain more caffeine and phenylpropanoids whereas for two varieties of *I. dumosa* the chemical markers were saponins and phenylpropanoids.

However, despite the clear metabolic separation of *Ilex* species, the identification of key metabolites contributing to the classification was difficult to implement, due to the overlapping of signals of the many similar analogues or glycosides of phenylpropanoids and saponins. In this context, 2D-NMR spectrum is required for the

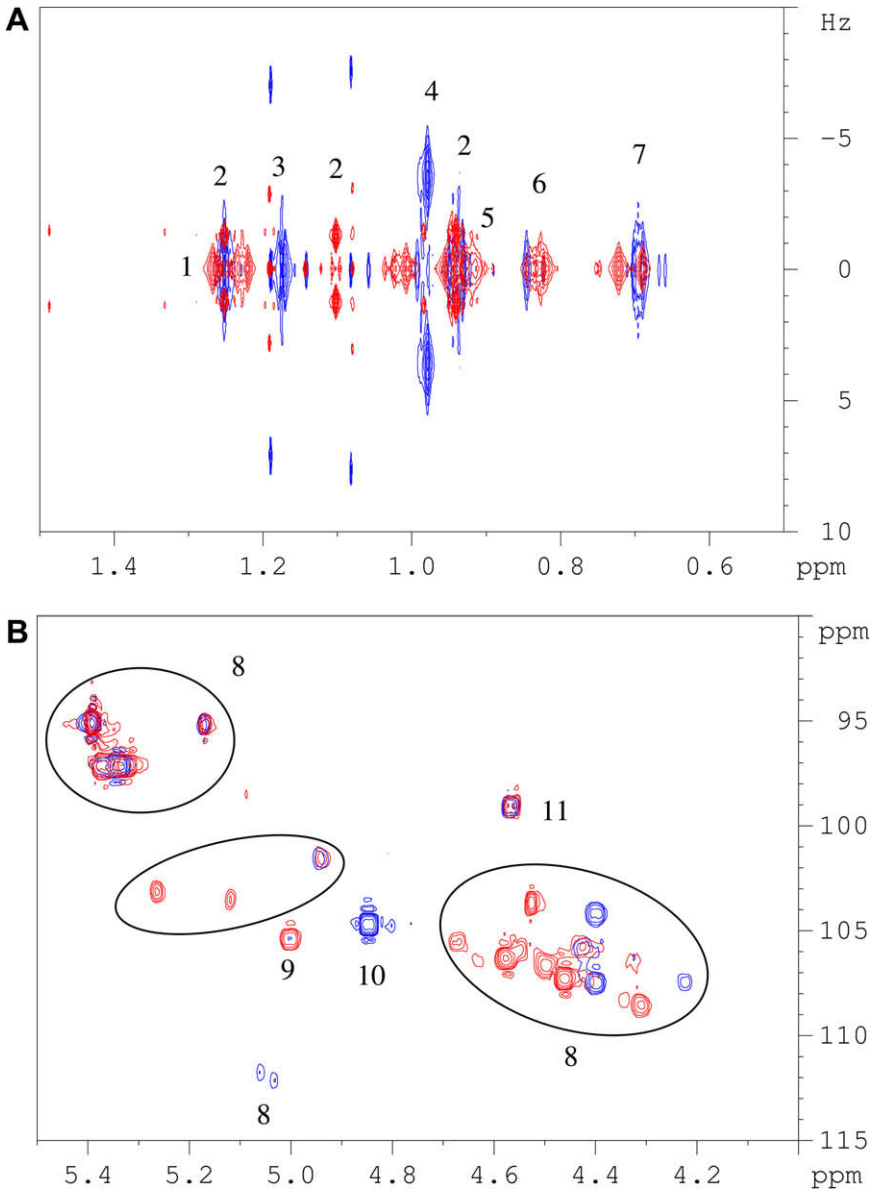


Fig. 7. 2D-*J*-resolved (A) and HSQC spectra (B) of *I. brevicuspis* (number of voucher specimen: 4) and *I. dumosa* var. *guaranina* (number of voucher specimen: 222) in the range of δ 0.5–1.5 for *J*-resolved, and δ 4.0–5.5 of ^{1}H and δ 90–115 of ^{13}C for HSQC. Blue: *I. brevicuspis*, red: *I. dumosa* var. *guaranina*. (1) H-27 of oleanane- or ursane-type saponins, (2) rhamnosyl H-6 of saponins or rutinoside of flavonoid glycosides. (3) H-23 of oleanane- or ursane-type saponins, (4) H-29 or H-30 of ursane-type saponins, (5) H-26 of oleanane- or ursane-type saponins, (6) H-25 of oleanane- or ursane-type saponins, (7) H-24 of oleanane- or ursane-type saponins, (8) HSQC correlation between anomeric protons and carbons of sugars in saponins, (9) HSQC correlation between glucosyl H-1 and C-1 rutin, (10) HSQC correlation between H-1 and C-1 of arbutin, (11) HSQC correlation between rhamnosyl H-1 and C-1 of rutin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identification of key distinguishing metabolites. Actually, groups both B and D showed a higher accumulation of saponins as compared to the other groups but the individual ^{1}H NMR resonances, particularly anomeric and methyl protons, differed between groups. Thus, it may be assumed that the metabolites which were shown to be increased were similar but not the same compounds.

2.3. Application of 2D-J-resolved and HSQC spectra to the Ilex species metabolomics

To solve the congestion of ^{1}H NMR, the use of ^{1}H–^{1}H 2D-*J*-resolved spectrum has been suggested because of its short measuring time and good quantitative features compared to other 2D-NMR experiments (Viant, 2003). In plant metabolomics it has been successfully applied to diverse plants such as *Nicotiana tabacum* (Choi et al., 2006), *Arabidopsis thaliana* (Hendrawati et al., 2006), *Brassica rapa* (Widarto et al., 2006; Liang et al., 2006) and *Panax ginseng* (Yang et al., 2006). In these applications, *J*-resolved spectra were applied not only for the identification of metabolites but also to improve spectral resolution by the projection to *F*-axis in which all the splitted signals become singlets (Viant, 2003; Choi et al., 2006; Hendrawati et al., 2006; Widarto et al., 2006; Liang et al., 2006; Yang et al., 2006).

Also, recently ^{1}H–^{13}C-heteronuclear single quantum coherence (HSQC) spectra has been applied in metabolomics studies (Hyberts et al., 2007; Lewis et al., 2007). Due to the inherent low sensitivity of ^{13}C, current metabolomics handling with mixtures could not use the ^{13}C derived metabolite information even though it is actually reflects the chemical structures more directly than that of ^{1}H NMR. Among the ^{13}C-related NMR techniques, HSQC shows rela-

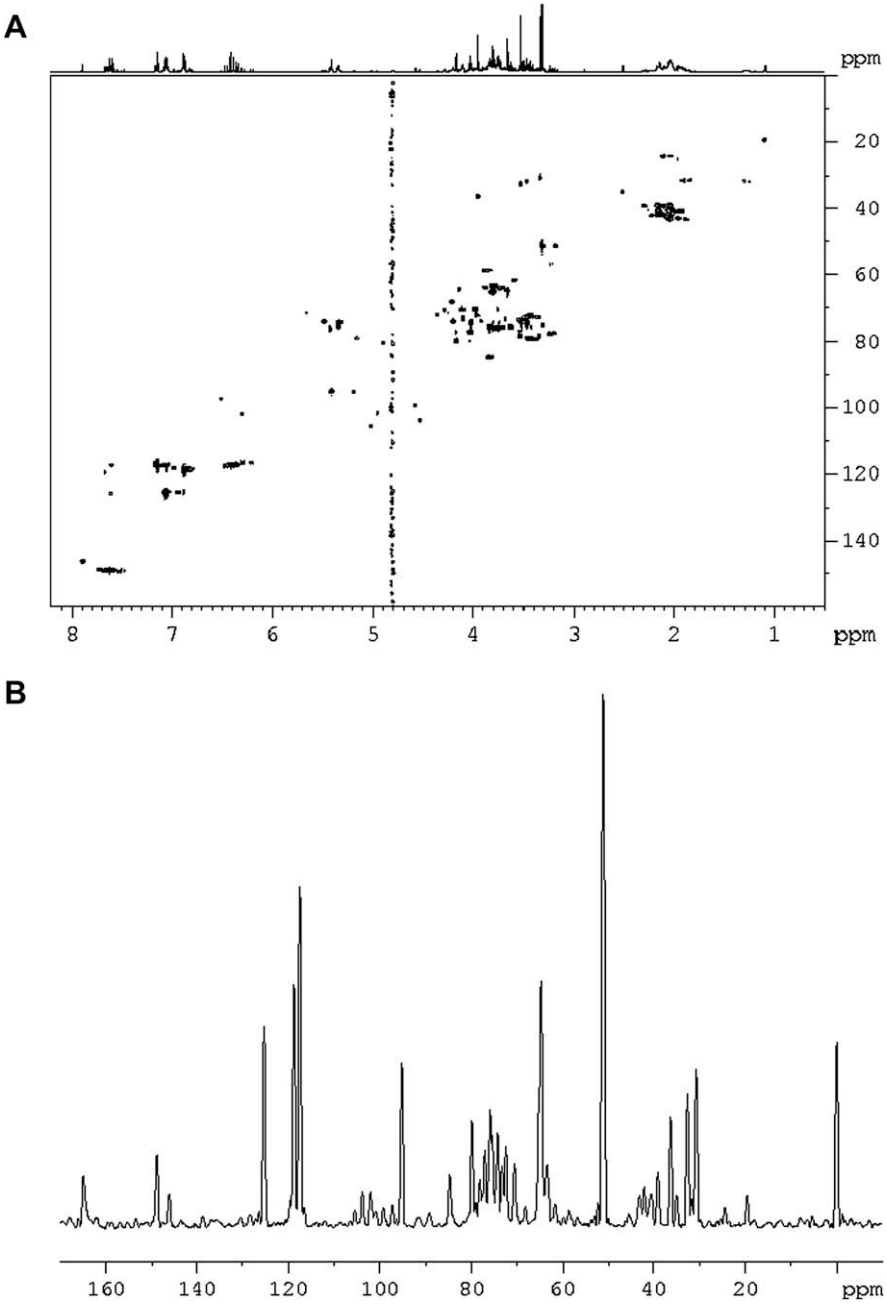


Fig. 8. Typical ^{1}H–^{13}C HSQC spectrum of *I. paraguariensis* (number of voucher specimen: 28) (A) and F_1-axis (^{13}C) projected 1D-HSQC spectrum (B).

tively higher resolution and quantitation features in shorter measuring time as compare to other ^{13}C-related NMR techniques (Hyberts et al., 2007; Lewis et al., 2007).

In this study *J*-resolved and HSQC were implemented for two purposes. Firstly, they were used for the metabolic fingerprinting of phenylpropanoids and saponins. In the metabolomic comparison of *Ilex* species and varieties, groups A and B showed a higher level of phenylpropanoids but ^{1}H NMR followed by multivariate data analysis did not afford any information on the individual phenylpropanoids. The same problem appeared in groups B and D with the *Ilex* saponins in this case. In an attempt to solve this, the 2D spectra of *J*-resolved and HSQC of these groups were compared.

Both *I. paraguariensis* and *I. dumosa* were found to contain higher levels of phenylpropanoids than other species in the PCA and PLS-DA results. To investigate the phenylpropanoid profile in detail, *J*-resolved and HSQC spectra were used for the ^{1}H and ^{13}C resonances of H-8′ and H-8″ in the range of δ 6.1–6.6 for ^{1}H and δ 90–130 for ^{13}C. In Fig. 6A, the *J*-resolved spectrum of *I. dumosa* var. *dumosa* showed extremely low intensities of H-8′ and H-8″ at δ 6.30 and δ 6.21 as compared to those of *I. paraguariensis*. Also, instead of the other two dicaffeoylquinic acids, isomers of chlorogenic acids such as 3-*O*- and 4-*O*-caffeoylquinic acids were found as major phenylpropanoids. In the HSQC spectrum, C-8′ and C-8″ resonances at δ 116.5 were clearly detected in *I. paraguariensis* but only trace signals were identified in *I. dumosa* (Fig. 6B).

In order to compare the saponin content of groups B and D which had showed to be high according to PCA results, the *J*-resolved and HSQC spectra of *I. dumosa* var. *guaranina* as a representative of group B and *I. brevicuspis* for group D were compared. Major *Ilex* saponins have been found to be mostly the ursane-type triterpenoids (Gosmann et al., 1989, 1995). In the *J*-resolved spectrum, *I. brevicuspis* showed the characteristic resonance of H-29 and H-30 of ursolic acid moiety around δ 9.7 (*d*, *J* = 7.2 Hz) (Fig. 7A). Interestingly, however, *J*-resolved spectrum of *I. dumosa* var. *guaranina* exhibited the characteristic singlet of methyls of the oleanane type saponins as major methyl resonances (Fig. 7A). This is consistent with previous reports on the constituents of *I. dumosa* in which diverse saponins of an oleanolic acid moiety rather than of ursolic acid present in other species have been reported (Heinzmann and Schenkel, 1995). Apart from this, *I. dumosa* was purported to have more diverse saponins than *I. brevicuspis* according to their HSQC spectrum. In the anomeric region of the HSQC spectrum, *I. dumosa* var. *guaranina* showed more varied anomeric sugar protons than *I. brevicuspis* (Fig. 7B).

As a further application of 2D-NMR spectra to metabolomics, the projection on F_1-axis (HSQC) and F_2-axis (*J*-resolved) were employed for further multivariate data analysis (Fig. 8), with the expectation of gathering more information that could not be obtained in ^{1}H NMR metabolomics thanks to the new variables with high resolution (*J*-resolved) and ^{13}C-information (HSQC). The classification based on PCA results obtained both from projected *J*-resolved (Fig. 9A) and HSQC (Fig. 9B) were the same as that obtained from ^{1}H NMR spectra except that the metabolome of *I. microdonta* appeared to be closer to group C (*I. integerrima*, *I. pseudobuxus* and *I. theezans*), which is different from group D according to the ^{1}H NMR results. However, PCA of the projected HSQC results showed exactly the same results as those obtained with ^{1}H NMR even for *I. microdonta*.

From the results of PCA, HCA, and PLS-DA based on ^{1}H NMR, *J*-resolved and HSQC spectra major metabolites for the chemical classification of 11 *Ilex* species and varieties were found to be secondary metabolites, including xanthines, phenylpropanoids, flavonoids, and triterpene saponins. Using the information, the metabolomic pattern of the key secondary metabolites of the *Ilex* species and varieties was expressed in a percentage form [(^{1}H NMR intensity of target resonance − mean value of the intensity

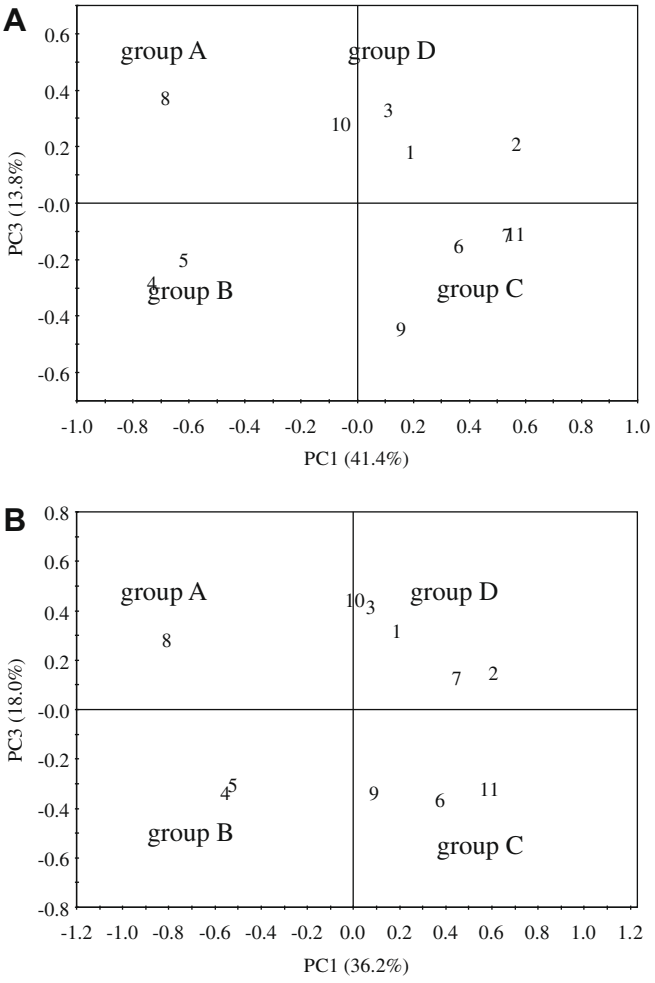


Fig. 9. Score plot of principal component analysis (PCA) obtained from F_2-axis-projected *J*-resolved (A) and F_1-axis-projected HSQC spectra (B). (1) *I. argentina*, (2) *I. brasiliensis*, (3) *I. brevicuspis*, (4) *I. dumosa* var. *dumosa*, (5) *I. dumosa* var. *guaranina*, (6) *I. integerrima*, (7) *I. microdonta*, (8) *I. paraguariensis* var. *paraguariensis*, (9) *I. pseudobuxus*, (10) *I. taubertiana*, (11) *I. theezans*.

in 11 *Ilex* species and verities) × 100] as can be observed in Fig. 10. The pattern is definitely consistent with the PCA, HCA and PLS-DA results. All 11 *Ilex* species and varieties are clearly divided into four groups; Group A (*I. paraguariensis*) for higher level of xanthines and phenolics including phenylpropanoids and flavonoids, group B (*I. dumosa* var. *dumosa* and *I. dumosa* var. *guaranina*) for saponins of an oleanolic acid moiety, group C (*I. integerrima*, *I. pseudobuxus* and *I. theezans*) for arbutin and phenylpropanoids (mostly dicaffeoylquinic acids) and group D (*I. argentina*, *I. brevicuspis*, *I. microdonta* and *I. taubertiana*) for the highest level of ursane-type saponins.

The classification of *I. brasiliensis* was not clear from PCA and HCA of NMR data. However, in the expression pattern of key metabolites, the species is more likely to belong to group C because of the higher amount of arbutin and lower amount of saponins.

3. Experimental

3.1. Plant materials

Dried plant leaves of 11 *Ilex* species were provided by the Estación Experimental Agraria of Cerro Azul (INTA) (Misiones, Argentina). The leaves were harvested two months prior to their use, dried for 3 min with a microwave (700 W), ground and preserved

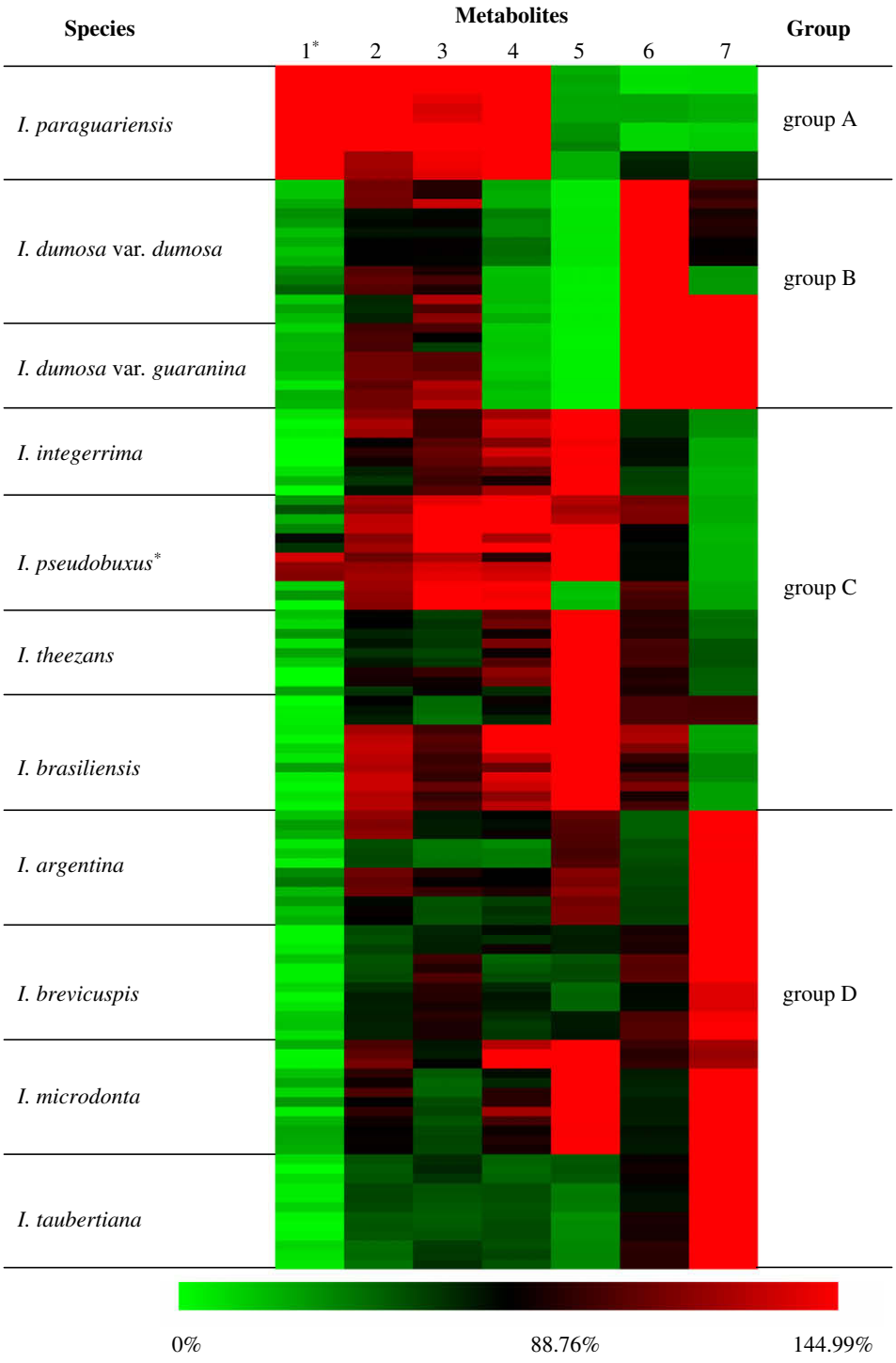


Fig. 10. Key metabolite expression (%) of 11 *Ilex* species and varieties. Expression percentage of metabolite = (^{1}H NMR intensity of target resonance − mean value of the intensity in 11 *Ilex* species and varities) × 100). (1) Xanthines (H-8 of caffeine at δ 7.89), (2) flavanoids (H-8 of rutin δ 6.53), (3) monocaffeoylquinic acids (H-8′ of 5-caffoyl quinic acid δ 6.36), (4) dicaffeoylquinic acids (H-8′ of 5-4,5-*O*-dicaffoyl quinic acid δ 6.30), (5) arbutin (H-2 and H-6 at δ 6.81), (6) total saponins including oleanane- and ursane-type triperpenes (H-26, at δ 0.92), (7) ursane-type triperpenes (H-29 and H-30, at δ 0.97). ∗: xanthine content of *I. pseudobuxus* was overestimated because it does not follow a normal distribution and only *I. paraguariensis* shows detectable amount of xanthines.

at –18 °C. Voucher specimens are preserved in the EEA Cerro Azul. Specifications of the plant materials evaluated in this study were reported in our previous study (Choi et al., 2005) (Table 1). CH_3OH-d_4 (99.9%) and D_2O (99.9%) were purchased from Cambridge Isotope Laboratories Inc. (Miami, FL, USA), and NaOD was from Cortec (Paris, France). Arbutin was obtained from Sigma (St. Louis, MO, USA).

3.2. Extraction of plant material

Each sample was freeze-dried. A sample of 50 mg of dry material was transferred to a 2 ml-microtube to which 1.5 ml of 50% CH_3OH-d_4 in buffer (90 mM KH_2PO_4 in D_2O) containing 0.05% trimethyl silyl propionic acid sodium salt (TMSP, w/v) were added. The mixture was vortexed at room temperature for 30 s, ultrasoni-

Table 1
Ilex species evaluated in this study (Choi et al., 2005).

Species	Region and year of seed-collection (number of voucher specimen)
Ilex argentina Lillo	Cerro san Javier, Tucumán, Argentina, 1991 (109) Acheral, Tucumán, Argentina, 1991 (111) Conception, Tucumán, Argentina, 1991 (112) Quebrada de San Lorenzo, Salta, Argentina, 1995 (207)
Ilex brasiliensis (Spreng) Loes.	Rio Branco do Sul, Paraná, Brazil, 1990 (59) Pto. Esperanza, Misiones, Argentina, 1990 (221) Reserva Biologica de Limoy, Paraguay, 1997 (226) Nueva Esperanza, Paraguay, 1997 (230)
Ilex brevicuspis Reissek	San Pedro, Misiones, Argentina, 1987 (4) Canoinhas, Brazil, 1989 (15) Clevelandia, Paraná, Brazil, 1991 (94) Veranopolis, Río Grande do Sul, Brazil, 1992 (119)
Ilex dumosa var. *dumosa* Reissek	Campo Viera, Misiones, Argentina, 1989 (7) Canoinhas, Santa Catarina, Brazil, 1989 (13) Tijucas do Sul, Paraná, Brazil, 1990 (55) Campo Bom, Río Grande do Sul, Brazil, 1992 (113)
I. dumosa var. *guaranina* Loes.	Pto. Esperanza, Misiones, Argentina, 1996 (222) Reserva Biologica de Limoy, Paraguay, 1997 (227) Hernandarias, Paraguay, 1998 (235) Hernandarias, Paraguay, 1998 (243)
Ilex integerrima (Vellozo) Reissek	Tijucas do Sul, Paraná, Brazil, 1990 (56) San Mateo do sul, Paraná, Brazil, 1990 (69) Iratí, Paraná, Brazil, 1990 (72) Teixeira Soares, Paraná, Brazil, 1990 (73)
Ilex microdonta Reissek	Sao Francisco de Paula, Río Grande do Sul, Brazil, 1992 (121) Sao Francisco de Paula, Río Grande do Sul, Brazil, 1992 (121a) Sao Francisco de Paula, Río Grande do Sul, Brazil, 1992 (121b) Parque Nacional Aparados da Serra, Río Grande do Sul, Brazil, 1992 (126)
Ilex paraguariensis var. *paraguariensis* St.-Hill.	Chapecó, Santa Catarina, Brazil, 1989 (28) San Antonio, Misiones, Argentina, 1989 (45) Teixeira Soares, Paraná, Brazil, 1990 (74) Ijuí, Río Grande do Sul, Brazil, 1993 (133)
Ilex pseudobuxus Reissek	Pontal do Sul, Paraná, Brazil, 1990 (67) Campo Bom, Río Grande do Sul, Brazil, 1992 (114) Torres, Río Grande do Sul, Brazil, 1992 (131) Tramandaí, Río Grande do Sul, Brazil, 1992 (132)
Ilex taubertiana Loes.	Sao Francisco de Paula, Río Grande do Sul, Brazil, 1992 (124) Sao Francisco de Paula, Río Grande do Sul, Brazil, 1992 (124a) Sao Francisco de Paula, Río Grande do Sul, Brazil, 1992 (124b) Sao Francisco de Paula, Río Grande do Sul, Brazil, 1992 (124c)
Ilex theezans Reissek	Major Viera, Río Grande do Sul, Brazil, 1989 (16) San Antonio, Misiones, Argentina, 1989 (46) Tijucas do Sul, Paraná, Brazil, 1990 (54) Veranopolis, Río Grande do Sul, Brazil, 1992 (118)

cated for 1 min, and centrifuged at 30,000 rpm at 4 °C for 20 min. The supernatant (700 μl) was taken for NMR analysis.

3.3. NMR analysis

1D-^{1}H NMR spectra, 2D-*J*-resolved spectra as well as ^{1}H–^{1}H homonuclear and inverse detected ^{1}H–^{13}C correlation experiments were recorded at 25 °C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz proton frequency) equipped with TCI cryoprobe and Z-gradient system. CD_3OD was used for internal lock purposes. For 1D-^{1}H NMR spectra a total of 32,768 data points were recorded covering a spectral window of 9615 Hz. One-hundred and twenty-eight scans of a standard one-pulse sequence with 30° flip angle for excitation and presaturation during 2.0 s relaxation delay with an effective field of $\gamma B_1 = 50$ Hz for suppression of the residual H_2O signal was employed (Price, 1999). Data was zero-filled to 65,536 points and an exponential window function with a line broadening factor of 0.3 Hz was applied prior to Fourier transformation. The resulting spectra were manually phased and baseline corrected, and referenced to internal TMSP at 0.0 ppm. For 2D-*J*-resolved NMR spectra (Aue et al., 1976) a data matrix of $62 \times 16{,}384$ data points covering 50×7739.4 Hz were acquired using 16 scans for each increment in F_1. Presaturation was applied during a relaxation delay of 1.5 s with an effective field of $\gamma B_1 = 50$ Hz. Data were zero-filled to $512 \times 32{,}768$ points prior to magnitude mode Fourier transformation with a sine shape window functions in both dimensions. The resulting frequency domain data were tilted by 45°, and then symmetrized along the F_2 dimension ($F_1 = 0$ Hz) and referenced according to internal TMSP. From the resulting 2D-*J*-resolved spectra 1D-projection along the F_2 dimension were extracted using the build-in positive projection routine in Topspin (version 2.1, Bruker Biospin). ^{1}H–^{1}H double-quantum filter correlation spectroscopy (DQF-COSY) spectra (Derome and Williamson, 1990) were acquired with presaturation ($\gamma B_1 = 50$ Hz) during a relaxation delay of 1.5 s. A data matrix of 1024×2048 points covering 7739.4×7739.4 Hz was recorded with eight scans for each increment. Data was zero-filled to 2048×2048 points prior to States-TPPI type 2D Fourier transformation and a sine bell shaped window function was applied in both dimensions. Coherence order selective gradient heteronuclear single quantum coherence (HSQC) spectra (Kay et al., 1992) were recorded for a data matrix of 256×2048 points covering 30182.7×7812.5 Hz with 64 scans for each increment. INEPT transfer delays were optimized for a heteronuclear coupling of 145 Hz and a relaxation delay of 1.5 s. was applied. Data was linear predicted in F_1 to 512×2048 using 32 coefficients and then zero-filled to 2048×2048 points prior to echo-anti echo type 2D Fourier transformation and a sine bell shaped window function shifted by $\pi/2$ in both dimensions was applied. 1D projection along the F_1-axis was extracted using the build-in positive projection tool of Topspin (version 2.1, Bruker Biospin). For heteronuclear multiple bond correlation (HMBC) spectra (Bax and Summers, 1986) a data matrix of 300×2048 points covering 33201.9×6265.6 Hz was recorded with 256 scans for each increment. A relaxation delay of 1.5 s and a coherence transfer delay optimized for a long range coupling of 8 Hz were applied. Data was linear predicted to 600×2048 points using 32 coefficients prior to echo-anti echo type 2D Fourier transformation and a sine bell shaped window function shifted by $\pi/2$ in the F_1 dimension and $\pi/6$ in the F_2 dimension was applied. The final spectrum was obtained by magnitude calculation along the F_2 dimension.

3.4. Data analysis

The ^{1}H NMR and the *J*-resolved projection spectra were automatically reduced to ASCII files using AMIX (version 3.7, Bruker Biospin). Spectral intensities were scaled to TMSP and reduced to integrated regions of equal width (0.04 ppm for ^{1}H- and projected *J*-resolved spectra, 0.5 ppm for projected HSQC spectra) corresponding to the region of δ 0.3–10.0. The region of δ 4.7–5.0 and was δ 3.28–3.34 excluded from the analysis because of the residual signal of H_2O and CH_3OH-d_4, respectively. Principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA), and hierarchical cluster analysis (HCA) were performed with the SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden). Both Pareto and unit variance (UV) scaling methods were applied to PCA and PLS-DA.

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References

Anderson, T., Fogh, J., 2001. Weight loss and delayed gastric emptying following a South American herbal preparation in overweight patients. J. Hum. Nutr. Diet. 14, 243–250.

Andrade, F.D.P., Piacente, S., Pizza, C., Vilegas, W., 2004. Arbutin-2′-sulphonyl from the infusion of *Ilex theezans* leaves. Fitoterapia 75, 782–784.

Ashihara, H., 1993. Purine metabolism and the biosynthesis of caffeine in maté leaves. Phytochemistry 33, 1427–1430.

Athayde, M.L., Coelho, G.C., Schenkel, E.P., 2000. Caffeine and theobromine in epicuticular wax of *Ilex paraguariensis* A. St.-Hil. Phytochemistry 55, 853–857.

Aue, W.P., Karhan, J., Ernst, R.R., 1976. Homonuclear broad band decoupling and two-dimensional *J*-resolved NMR spectroscopy. J. Chem. Phys. 64, 4226–4227.

Bailey, N.J.C., Sampson, J., Hylands, P.J., Nicholson, J.K., Holmes, E., 2002. Multi-component metabolic classification of commercial feverfew preparations via high-field ^{1}H-NMR spectroscopy and chemometrics. Planta Med. 68, 734–738.

Baltassat, F., Darbour, N., Ferry, S., 1984. Etude du contenu purique de drogues a caféine: I. – Le maté: *Ilex paraguariensis* Lamb. Planta Med. Phytother. 18, 195–203.

Bax, A., Summers, M.F., 1986. ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108, 2093.

Cardozo Jr., E.L., Filho, O.F., Filho, L.C., Ferrarese, M.L.L., Donaduzzi, C.M., Sturion, J.A., 2007. Methylxanthines and phenolic compounds in mate (*Ilex paraguariensis* St. Hil.) progenies grown in Brazil. J. Food Compos. Anal. 20, 553–558.

Choi, Y.H., Kim, H.K., Hazekamp, A., Erkelens, C., Lefeber, A.W.M., Verpoorte, R., 2004. Metabolomic differentiation of *Cannabis sativa* cultivars using ^{1}H NMR spectroscopy and principal component analysis. J. Nat. Prod. 67, 953–957.

Choi, Y.H., Sertic, S., Kim, H.K., Wilson, E.G., Michopoulosa, F., Lefeber, A.W.M., Erkelens, C., Kricun, S.D.P., Verpoorte, R., 2005. Classification of *Ilex* species based on metabolomic fingerprinting using nuclear magnetic resonance and multivariate data analysis. J. Agric. Food Chem. 53, 1237–1245.

Choi, Y.H., Kim, H.K., Linthorst, H.J.M., Hollander, J.G., Lefeber, A.W.M., Erkelens, C., Nuzillard, J.-M., Verpoorte, R., 2006. NMR metabolomics to revisit the tobacco mosaic virus infection in *Nicotiana tabacum* leaves. J. Nat. Prod. 69, 742–748.

Clifford, M.N., Ramirez-Martinez, J.R., 1990. Chlorogenic acids and purine alkaloids contents of Maté (*Ilex paraguariensis*) leaf and beverage. Food Chem. 35, 13–21.

Daolio, C., Beltrame, F.L., Ferreira, A.G., Cass, Q.B., Cortez, D.A.G., Ferreira, M.C., 2008. Classification of commercial catuaba samples by NMR, HPLC and chemometrics. Phytochem. Anal. 19, 218–228.

Derome, A., Williamson, M., 1990. Rapid pulsing artifacts in double-quantum-filtered COSY. J. Magn. Reson. 88, 177–185.

Filip, R., Lopez, P., Coussio, J.D., Ferraro, G., 1998. Mate substitutes or adulterants: study of xanthine content. Phytother. Res. 12, 129–131.

Filip, R., Lotito, S.B., Ferraro, G., Fraga, C.G., 2000. Antioxidant activity of *Ilex paraguariensis* and related species. Nutr. Res. 20, 1437–1446.

Filip, R., Lopez, P., Giberti, G., Coussio, J., Ferraro, G., 2001. Phenolic compounds in seven South American *Ilex* species. Fitoterapia 72, 774–778.

Galle, F.C., 1997. Hollies: The Genus *Ilex*. Timber Press, Portland.

Giberti, G.C., 1989. Los parientes silvestres de la yerba mate y el problema de su adulteración. Dominguezia 7, 1–22.

Gonzalez, A., Ferreira, F., Vazquez, A., Moyna, P., Alonso Paz, E., 1993. Biological screening of Uruguayan medicinal plants. J. Ethnopharmacol. 39, 217–220.

Gorzalczany, S., Filip, R., Del Rosario Alonso, M., Miño, J., Ferraro, G.E., Acevedo, C., 2001. Choleretic effect and intestinal propulsion of "mate" (*Ilex paraguariensis*) and its substitutes or adulterants. J. Ethnopharmacol. 75, 291–294.

Gosmann, G., Schenkel, E.P., Seligmann, O., 1989. A new saponin from mate, *Ilex paraguariensis*. J. Nat. Prod. 52, 1367–1370.

Gosmann, G., Guillaume, D., Taketa, A.T.C., Schenkel, E.P., 1995. Triterpenoid saponins from *Ilex paraguariensis*. J. Nat. Prod. 58, 438–441.

Gottleib, A.M., Giberti, G.C., Poggio, L., 2005. Molecular analyses of the genus *Ilex* (Aquifoliaceae) in southern South America, evidence from AFLP and ITS sequence data. Am. J. Bot. 92, 352–369.

Gugliucci, A., Menini, T., 2002. The botanical extracts of *Achyrocline sauteroides* and *Ilex paraguariensis* prevent methylglyoxal-induced inhibition of plasminogen and antithrombin III. Life Sci. 72, 279–292.

Heinzmann, B.M., Schenkel, E.P., 1995. Saponins from *Ilex dumosa*. J. Nat. Prod. 58, 1419–1422.

Hendrawati, O., Yao, Q., Kim, H.K., Linthorst, H.J.M., Erkelens, C., Lefeber, A.W.M., Choi, Y.H., Verpoorte, R., 2006. Metabolic differentiation of *Arabidopsis* treated with methyl jasmonate using nuclear magnetic resonance spectroscopy. Plant Sci. 170, 1118–1124.

Hyberts, S.G., Heffron, G.J., Tarragona, N.G., Solanky, K., Edmonds, K.A., Luithardt, H., Fejzo, J., Chorev, M., Aktas, H., Colson, K., Falchuk, K.H., Halperin, J.A., Wagner, G., 2007. Ultrahigh-resolution ^{1}H–^{13}C HSQC spectra of metabolite mixtures using nonlinear sampling and forward maximum entropy reconstruction. J. Agric. Food Chem. 129, 5108–5116.

Kay, L.E., Keifer, P., Saarinen, T., 1992. Pure absorbtion gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. J. Am. Chem. Soc. 114, 10663–10665.

Kim, H.K., Choi, Y.H., Erkelens, C., Lefeber, A.W.M., Verpoorte, R., 2005. Metabolic fingerprinting of *Ephedra* species using ^{1}H-NMR spectroscopy and principal component analysis. Chem. Pharm. Bull. 53, 105–109.

Kraemer, K.H., Taketa, A.T.C., Schenkel, E.P., Gosmann, G., Guillaume, D., 1996. Matesaponin 5, a highly polar saponin from *Ilex paraguariensis*. Phytochemistry 42, 1119–1122.

Lewis, I.A., Schommer, S.C., Hodis, B., Robb, K.A., Tonelli, M., Westler, W.M., Sussman, M.R., Markley, J.L., 2007. Method for determining molar concentrations of metabolites in complex solutions from two-dimensional ^{1}H–^{13}C NMR spectra. Anal. Chem. 79, 9385–9390.

Liang, Y.-S., Choi, Y.H., Kim, H.K., Linthorst, H.J.M., Verpoorte, R., 2006. Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by two dimensional NMR spectroscopy and multivariate analysis. Phytochemistry 67, 2503–2511.

Maria, A., Martinez, D.P., Pelotto, J.P., Basualdo, N., 1997. Distribution of flavonoid aglycones in *Ilex* species (Aquifoliaceae). Biochem. Syst. Ecol. 25, 619–622.

Mazzafera, P., 1994. Caffeine, theobromine and theophylline distribution in *Ilex paraguariensis*. Rev. Bras. Fisiol. Veg. 6, 49–151.

Mucillo-Baisch, A.L., Johnston, K.B., Paganini-Stein, F.L., 1998. Endothelium-dependent vasorelaxing activity of aqueous extracts of *Ilex paraguariensis* on mesenteric arterial bed of rats. J. Ethnopharmacol. 60, 133–139.

Niemann, G.J., Baas, W.J., 1985. The composition of the lipid constituents of *Ilex aquifolium* L. (Aquifoliaceae) in relation to the age of the leaf. J. Plant Physiol. 118, 209–218.

Noud, P.C., Martinez, M.A.D.P., Loizeau, P.A., Spichiger, R., Andrews, S., Manen, J.F., 2000. Molecular phylogeny and biogeography of the genus *Ilex* L. (Aquifoliaceae). Ann. Bot. London 85, 111–122.

Pauli, G.F., Poetsch, F., Nahrstedt, A., 1998. Structure assignment of natural quinic acid derivatives using proton nuclear magnetic resonance techniques. Phytochem. Anal. 9, 177–185.

Peluso, G., Feo, V., Simone, F., Bresciano, E., Vuotto, M.L., 1995. Studies on the inhibitory effects of caffeoylquinic acids on monocyte migration and superoxide anion production. J. Nat. Prod. 58, 639–646.

Price, W.S., 1999. Water signal suppression in NMR spectroscopy. Ann. Rep. NMR Spectrosc. 38, 289–354.

Ricco, R.A., Wagner, M.L., Gurni, A., 1991. Estudio comparativo de flavonoides en seis especies austrosudamericanas de genero *Ilex*. Acta Farm. Bonaerense 10, 29–35.

Schenkel, E.P., Gosmann, G., Montanha, J.A., Heizmann, B.M., Athayde, M.L., Taketa, A.T.C., Pires, V.S., Guillaume, D., 1997. Saponins from Maté (*Ilex paraguariensis*) and other South American *Ilex* species: ten years research on *Ilex* saponins. Ciên. Cult. 49, 359–363.

Taketa, A.T., Schmittmann-Schlager, T., Guillaume, D., Gosmann, G., Schenkel, E.P., 2000. Triterpenoid glycosides and a triterpene from *Ilex brevicuspis*. Phytochemistry 53, 901–904.

Viant, M.R., 2003. Improved methods for the acquisition and interpretation of NMR metabolomic data. Biochem. Biophys. Res. Commun. 310, 943–948.

Widarto, H.T., Van der Meijden, E., Lefeber, A.W.M., Erkelens, C., Kim, H.K., Choi, Y.H., Verpoorte, R., 2006. Metabolomic differentiation of *Brassica rapa* leaves attacked by herbivore using two dimensional nuclear magnetic resonance spectroscopy. J. Chem. Ecol. 32, 2417–2428.

Yang, S.Y., Kim, H.K., Lefeber, A.W.M., Erkelens, C., Angelova, N., Choi, Y.H., Verpoorte, R., 2006. Application of two dimensional nuclear magnetic resonance spectroscopy to quality control of ginseng commercial products. Planta Med. 72, 364–369.