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Populus nigra L. bud absolute: a case study for a strategy of analysis of natural complex substances

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Abstract

The new European regulations (e.g. REACH) require that Natural Complex Substances such as essential oils, absolutes, concretes and resinoids are registered. This need implies that the chemical composition of these complex mixtures is characterized as exhaustively as possible in view of defining their toxicological risk. This study proposes a strategy of analysis to be applied to the chemical characterization of poplar absolute as an example of Natural Complex Substances of vegetable origin. In the first part, the proposed strategy is described and the advantages and the limitations related to the combination of conventional analytical techniques such as GC without and with sample derivatization and HPLC are critically discussed. In the second part, the qualitative data obtained with GC and HPLC analysis of poplar bud absolute confirm the sample complexity which mainly consists of phenolic components. Fourteen compounds (i.e. phenolic acids, phenylpropanoids and flavonoids) were then chosen as markers representative of the main classes of components characterizing poplar bud absolute. The marker quantitation carried out by GC-SIM-MS and HPLC-PDA analyses gives similar results confirming the reliability of both techniques. These results demonstrate that conventional analytical techniques can positively and effectively contribute to study the composition of natural complex substances, i.e. matrices for which highly effective separation is necessary, consisting them mainly of isomers or homologous components. The combination of GC and HPLC techniques is ever more necessary for routine quality control when conventional instrumentations are used.

Introduction

The new regulations, in particular those introduced by the European Community (e.g. REACH), require that Natural Complex Substances (NCS) such as essential oils, absolutes, concretes and resinoids produced or imported in amounts above 1 tonn/year in the EC has to be registered together with their toxicological properties and possibly with their environmental impact in order to inform consumers of risk assessment [1]. NCS registration is not easy because of their complexity, since an as exhaustive as possible chemical characterization is mandatory to define its toxicological risk. According to the ISO norms an absolute is "a product with odour obtained from a concrete or a resinoid by extraction with ethanol at room temperature, cooled and filtered to remove the waxes; the ethanol is then removed by distillation" and a concrete (or a resinoid) is " an extract with a characteristic odour from a fresh (or dried) plant raw material by a non aqueous solvent" (in general hydrocarbons) [2].

In general, an absolute contains hundredths of lipophilic compounds of different volatility that make complex to investigate its chemical composition, as demonstrated by the limited number of articles available [3,4]. An *ad hoc* strategy to study absolute composition and its biological activity is therefore necessary. This article proposes a possible approach to investigate absolute composition, to define its markers, with the main aim to make it applicable to routine control. This method consists of the combination of a) a non-targeted step that involves an exhaustive analysis to obtain qualitative and semi quantitative composition (mainly expressed as relative percent abundance) enabling the definition of the main classes and/or groups of compounds in the absolute under investigation, followed by b) a targeted step to quantify a number of representative markers selected within the most significant groups in the total extract. Poplar bud (*Populus nigra* L. -Salicaceae) absolute has here been used to develop and test the proposed strategy. This absolute is widely used in flavor, fragrance and cosmetic industries for its fragrance and for the antioxidative properties due to the presence of phenolic compounds [5,6].

The genus *Populus* (Salicaceae family) consists of about 30 species [7], growing in southern and central Europe, in central Asia, Siberia and North America, characterized and differentiated by the presence of different flavonoids, phenolic derivatives and terpenoids in particular in the young leaves, buds and bud exudates [8-11]. The species belonging to this genus can chemically be distinguished on the basis of their composition [8-12]. *Populus nigra* L. buds have been described mainly to contain terpenoids (in particular mono and sesquiterpenoids) and phenolic compounds (phenols, phenolic acids, phenylpropanoids and different subgroups of flavonoids) [[8-10,12-14] and reference cited therein].

This study discusses critically the proposed strategy to analyze a NCS of vegetable origin with conventional techniques in view of its application in routine quality control and the results obtained when it is applied to the poplar bud absolute.

Experimental

Plant Material and chemicals

Poplar absolute from *Populus nigra* L. buds (lots 221265 and 1772363) was produced by Robertet SA (Grasse, France). Three samples for each lots were analyzed.

Pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane and benzoic acid, caffeic acid, *trans-p*-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethoxy cinnamic acid, *trans*-cinnamic

acid, *p*-methoxy-cinnamic acid, and 1,1-dimethylallyl caffeate (purity > 98%) were from Sigma-Aldrich (St. Louis MO, USA). Chrysin, galangin, pinocembrin, pinostrobin, tectochrysin (purity >99%) were from Extrasynthese (Genay, France). HPLC and analytical grade solvents were from Carlo Erba Reagenti (Rodano, Italy).

Vacuum molecular distillation

Poplar bud absolute (300 g) was submitted to vacuum molecular distillation in a KDL4 system (Leybold-Heraeus, Oetikon, Switzerland) for 150 min in Robertet laboratories in Grasse (France). Initial temperature: 60°C, cooling temperature: 50°C. Distillation occurred at 130°C and 1×10^{-3} bar under stirring (250 rpm). Molecular distillation provided three fractions of different volatility: 1) a volatile fraction (VF) collected in the first condenser, 2) a distilled fraction (DF) collected in the second condenser, and 3) a residue fraction (RF) containing the components not distilled under the conditions applied. The resulting fractions were preliminarily analyzed by GC–FID, GC-MS , HPLC-PDA, and HPLC-PDA-MS (see previous sections).

Sample and fraction processing

a) *Volatile fraction (VF)*. On the basis of the preliminary GC-FID and GC-MS analysis results, the oily yellow VF fraction was submitted to a 1/1 v/v basic extraction with $NaHCO_3$ (10%) to separate acidic components. Free acids were restored by acidifying the aqueous phase and extracting them with methylene chloride. The acid-free VF was submitted to column chromatography on Silica gel with petroleum ether/ethyl acetate varying from 100/0 to 70/30.

b) *Distilled fraction (DF)*. The light yellow gummy DF sample was dissolved in ethyl acetate and submitted to the same treatments as for VF fraction (i.e. deacidification), and column chromatography under the same conditions.

c) *Fraction and subfraction derivatization*. Pyridine (80 μl) and bis(trimethylsilyl)-trifloroacetamide (BSTFA) with 1% trimethylchlorosilane (120 μl) were added to 2 mg of each fraction (VF, DF and RF and related subfractions) and heated at 60°C for 30 min to derivatize hydroxylated and carboxylic components to the corresponding trimethylsilyl derivatives (TMS).

GC-FID and GC-MS analysis

GC analyses were carried out on a Shimadzu 2010 GC-FID system and a Shimadzu QP2010 plus GC-MS system, both provided with an AOC-20i automatic injector, and with Shimadzu GC Solution 2.53SU1 software and Shimadzu GCMS Solution 2.51 software, respectively (Shimadzu, Milan, Italy).

GC-FID-MS analyses were carried out on a Mega 5 column (95% polydimethyl-siloxane, 5% phenyl) 25 m x 0.25 mm d_c x 0.25 μm d_f, from MEGA (Milano – Italy).

GC-FID and GC-MS conditions: injection mode: split; split ratio: 1:20; injection volume: 1μl. Temperatures: injector: 250 °C, FID: detector: 280 °C, MS: transfer line: 280 °C; ion source: 200 °C; carrier gas: He, initial flow-rate 1.0 ml/min in costant linear velocity mode. Temperature programme: from 50 °C (1 min) to 280 °C (10

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min) at 3 °C/min. The MS operated in electron impact ionization mode (EI) at 70 eV, at a scan rate of 1111 u/s with a mass range of 35–750 m/z, suitable to cover the full fragmentation pattern of all analytes investigated in full scan mode; an interval time of 0.2 s was applied in SIM mode to three diagnostic ions for each standard component.

Absolute volatile components were identified by comparison of both their linear retention indices (I^{T}_{S}), calculated *versus* a C_9-C_{26}, C_{28}, C_{30}, C_{32}, C_{34} hydrocarbon mixture, and their mass spectra to those of authentic samples, or data from home-made or from commercial libraries or reported in the literature [12, 15-18]

Quantitative GC-SIM-MS analysis. Suitable amounts of caffeic acid, *trans-p*-coumaric acid, ferulic acid, isoferulic acid, benzoic acid, 1,4-dimethoxycinnamic acid, *trans*-cinnamic acid, *p*-methoxy-cinnamic acid, 1,1 dimethylallyl caffeate, chrysin, galangin, pinocembrin, pinostrobin, tectochrysin were diluted with acetone to obtain 5 different concentrations in the range of 0.05 to 0.5 μg/μl for each marker. 1ml of standard solution for each concentration was evaporated to dryness and derivatized to the corresponding trimethylsilyl derivatives (TMS) as reported above. Each concentration was derivatized in triplicate. Calibration curves were built by analyzing the resulting TMS-derivatized standard solutions three times by GC-MS in SIM mode under the conditions reported above.

HPLC-PDA-MS analysis

DF and RF were analyzed by a Shimadzu LC-MS 2010EV system equipped with a photodiode detector SPD-M20A (Shimadzu, Dusseldorf Germany) in series to a single quadrupole MS system provided with orthogonal atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources. An Ascentis Express C18 column (150 x 4.6 mm i.d., 2.7 μm), (Supelco, Bellefonte, PA) was used. Analysis conditions were: temperature: 40°C; mobile phase: eluent A: water/0.1% formic acid; eluent B: methanol/0.1% formic acid; mobile phase gradient was as follows: 40% B for 10 min, 40-48% B in 10 min, 48-60% B in 10 min, 60% for 10 min, 60-90% B in 40 min and 90% B for 10 min. Injection volume: 5 μl, flow rate: 0.4 ml/min. UV spectra were acquired in the 210-450 nm wavelength range and the resulting chromatograms were integrated at different wavelengths in function of the UV absorption maxima of each component. MS operative conditions: ESI temperature: 200°C; nebulizer gas flow rate: 1.5 ml/min; curve desolvation line (CDL) temperature: 250°C. Mass spectra were acquired both in positive and in negative full-scan mode in the range of 100-700 m/z, with a scan range of 1000 u/s.

Quantitative HPLC-PDA analysis. Suitable amounts of caffeic, *trans-p*-coumaric, ferulic, isoferulic, benzoic, dimethyl caffeic, *trans*-cinnamic and *p*-methoxy-cinnamic acid, caffeic acid 1,1-dimethyl allyl ester, chrysin, galangin, pinocembrin, pinostrobin, tectochrysin were dissolved in methanol to obtain concentrations from 1 to 50 μg/ml of each marker. Calibration curves were built by analyzing the resulting standard solutions three times by HPLC-PDA.

Results and discussion

This section consists of two main parts: 1) a critical discussion of the strategy to adopt for NCS analysis; 2) the results obtained by its application to the poplar bud absolute.

Strategy of analysis

As already mentioned, this strategy combines a non-targeted step to define as exhaustively as possible qualitative composition and to measure the indicative percentage abundance of the main groups of compounds in the absolute under investigation, and a targeted step to select and quantify a number of representative markers suitable to characterize it Moreover, in view of the use of this strategy in quality control, techniques conventionally available in routine laboratory are here adopted. The strategy of analysis of course depends on the complexity of the specific absolute, although, in general, because of the number of components that accounts for some hundredths, the original sample has to be "simplified" through a preliminary fractionation to meet the aim to study its composition exhaustively. The process to obtain absolutes excludes both highly apolar and polar compounds (e.g. waxes, fats, glycosides etc.) while it does not discriminate between high and low volatility compounds. These sample characteristics mean that, in general, an absolute is not analyzable in a single run as such and/or with a single chromatographic technique (e.g. HPLC or GC). One of the approaches that leaves out of the nature of the absolute components is a fractionation on the basis of their volatility through a vacuum or molecular distillation, the vacuum being necessary to avoid high temperatures inducing artefact formation and limiting component thermal degradation. This operation should provide fractions of homogeneous volatility and therefore analyzable *in toto* with the most appropriate technique. Further fractionation based on acid/basic extractions or on polarity by SPE, column or flash chromatography, preparative LC can afterward be carried out to simplify ever more the composition of the fractions from distillation or to isolate unknown markers (where present) and elucidate their structures by spectroscopic techniques (e.g. NMR). Molecular distillation of the investigated poplar bud absolute has produced three fractions; as reported below it met the aim to simplify sample composition, since the first fraction consisted of volatiles (volatile fraction, VF) mainly sesquiterpenoids, the second one of medium volatility components (distilled fraction, DF) containing above all phenolic derivatives and a residue containing high boiling compounds (residue fraction, RF).

Several studies have demonstrated how important is to combine results from GC and HPLC in the quali-quantitative analyses of natural complex mixtures as an approach to overcome the intrinsic limitations of these two techniques [19-22]. However, the conventional analysis of an absolute such as that of poplar buds is conditioned by some objective limits of these two separation techniques. GC is limited in the analysis of low volatility compounds, although its use can be extended to medium-high boiling and medium polarity compounds by modifying their volatility through suitable derivatisation reactions. The role of GC-MS including derivatization in plant metabolite profiling was critically discussed by Halket et al. [23] and Fiehn [24]. More recently, Isidorov and Szczepaniak [18] reported the retention indices of 389 biologically and environmentally important organic compounds determined with moderately polar columns (polydimethylsiloxane-5%-phenyl) most of them derivatized to the corresponding trimethylsylil derivatives (TMS) with pyridine and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane and evaluated I^T_S reliability both of underivatized and of the corresponding derivatized analytes and the factors influencing their reproducibility. Analyte derivatisation offers: 1) an FID response to analytes only depending on their amounts at least within a

homogeneous group of compounds, 2) the possibility to apply I^T_S to locate (and identify) analytes in the total chromatogram; 3) mass spectra with diagnostic and reproducible fragmentation patterns, 4) improvement of the peak shape of components interacting with the stationary phase producing leading or tailing peak distortion (in this case e.g. benzoic acid), thus increasing the reliability of quantitative data. On the other hand, derivatization of low volatility components requires: a) a correct application of percent normalization by using an internal standard and FID response factors [25], b) availability of reference standards for a correct identification and quantitation, c) careful tuning of derivatization conditions to achieve high yield and avoid multiple products due to incomplete derivatization of molecules containing hydroxyls with a different reactivity, that can make the chromatogram ever more complex, and d) possibility of alteration of the gas chromatographic (injector, liner activity, column efficiency) and MS performance due to possible decomposition of reagents and derivatized analyte(s). Lastly, GC-MS can exploit commercially, literature and/or in-house available linear retention index (I^T) collections [15, 18, 26-28] interactively or not combined to mass spectral libraries as a tool for component identification, unlike what happens in HPLC-MS (or UV).

HPLC also shows some limitations in the analysis of highly complex mixtures although its most recent advances (Ultra High Pressure LC (UHPLC) and the introduction of evoluted IT or TOF analyzers and hybrid systems [[29,30] and references cited therein]) have dramatically increased its performance. In particular a) HPLC-UV is unable to detect compounds without chromophoric groups; moreover, its response depends on analyte molar absorbivity that is structure-specific, thus altering the relative ratios between the sample components and making impossible to establish a correct ratio between peak abundances and, as a consequence, a quantitation (or at least semi-quantitation) without reference standards, and b) HPLC-MS gives responses that are again influenced by i) the analyte structure, whose fragmentation also depends on the adopted HPLC-MS interface and ion source, ii) the analyte ionization rate that can vary depending on structures, iii) the nature of the analyzer, and iv) the limitations in mobile phase composition and flow-rate that can affect HPLC separation flexibility. .

The analysis of natural complex substances and in particular of poplar bud absolute implies the fundamental complementary use of all discussed techniques since: i) GC-FID or MS without derivatisation is always necessary not only to provide with a characterization of volatiles and their rate but also as a reference GC profile to be compared to that of the derivatized sample as here shown for sesquiterpene hydrocarbon identification and flavonoid quantitation (see next paragraphs), ii) GC-MS with derivatisation makes analyzable by GC poorly or not volatilizable or medium-high polarity compounds contributing to their identification and making possible their quantitation, and iii) HPLC-UV and MS are indispensable for identification and quantitation of non-volatile compounds. These techniques could indifferently be used for quantitation of selected markers provided that standards are available and the investigated peaks are correctly eluted and separated, thus making possible a cross control of the results. In addition, GC-FID can provide reliable semi-quantitation (% normalization) and peak relative abundance provided that an internal standard and average FID response factors are available. GC-FID without derivatization is the preferred technique for semi-quantitation preliminary but important indispensable step for sample comparison.

Qualitative and Quantitative analyses of poplar bud absolute

This study aims to characterize the chemical composition of the absolute obtained from *Populus nigra* L buds. Fig. 1 reports the GC-MS profiles as such and after derivatization of the total absolute and Fig. 2 the

corresponding HPLC-PDA profile. Poplar bud absolute was first submitted to molecular distillation from which three fractions of homogeneous volatility were obtained: VF (representing about 4% of the total absolute), DF (about 20 %) and RF (about 75 %). Fig S1 and S2 (supplementary data files) show the effectiveness of the molecular distillation. The repeatability resulting from molecular distillation of the six samples from the two lots of poplar bud absolute was good giving RSD% of 10% for VF, 7% for DF and 4% for RF. Each fraction was then deacidified, and submitted to a further fractionation by silica column chromatography in order to simplify furthermore the chemical profiles and to facilitate component location and identification. The three main fractions and those resulting from the following treatments were all analyzed by GC-FID-MS as such and/or after derivatization with BSTFA and by HPLC-PDA-MS.

GC-MS analysis

 215 and 170 peaks were respectively counted in GC-FID-MS absolute profiles with and without derivatization. Tables 1a and 1b report the list of the identified components in the total absolute and in the three fractions obtained from molecular distillation analysed by GC-FID-MS with (1a) and without (1b) derivatization. Both tables report the components detected by GC-FID-MS analysis together with their I^T_S. The components listed in table 1a and 1b without a percent value were not found in the underivatized and/or derivatized total chromatograms because present in very low amounts and only detected in the absolute fractions. In addition, in table 1a, the number of TMS-derivatized groups is reported for each derivatizable component. The supplementary files S3and S4 report the mass spectra of the main unidentified components (> 0.2%) in the GC-FID-MS profiles with and without the derivatization procedure together with their I^T.

The identification was confirmed with the co-injection of authentic standards (when available) after derivatization. VF was mainly characterized by a mixture of terpenoids, phenolic acids, alcohols and esters, fatty acids and related esters with I^T_S calculated *vs* an homologous series of hydrocarbons up to 2200 on polydimethylsiloxane, 5% phenyl as GC stationary phase, and molecular weights (m.w.) up to around 300 (ethyl linoleate m.w. 308 (55)); DF mainly consisted of flavonoid aglycones, cinnamic acid esters, phenolic acids with I^T_S up to 3200 and a M.W. up to about 450 (triadocontane, $C_{32}H_{66}$); RF mainly contained phenolic compounds. VF fraction contains several sesquiterpene hydrocarbons (most of them present in very low amounts or traces), sesquiterpenoids (α- and β-eudesmol), phenols and phenolic acids and derivatives (the main one of them being benzoic acid (9), in the total absolute) together with glycerol (14) and catechol (15)), fatty acids and related esters (hexadecanoic acid (50) and ethyl hexadecanoate (palmitate) (48). Sesquiterpene hydrocarbons always present as minor or trace components were mainly identified with the underivatized GC analysis being not detectable in the absolute derivatized GC profile and difficult to detect in the derivatized VF fraction because their spectra were not sufficiently significant due to the higher noise. DF fraction mainly consists of homologous series of cynnamic acids (*p*-coumaric (47) and *p*-methoxy-cynnamic (46) acids) and their esters (3-methyl-2-butenyl-*p*-coumarate (59), dimethyl allyl caffeoate (70), benzyl-*p*-coumarate (72), benzyl caffeoate (81)) and a series of flavonoids and derivatives (pinostrobin (67,71), pinocembrin (73), pinobanksin (74), chrisyn (75, 82), galangin (80,84) in different abundances) all without hydroxyls on the B-ring. Flavonoids deriving from *p*-coumaroyl-CoA/naringeninchalcone and caffeoyl-CoA/eriodictyol chalcone biosynthetic pathways (i.e. with hydroxyls in ring B) already identified in *P. nigra* [8, 31-33] were not found most probably because they are not extracted by the apolar solvent in absolute preparation due to their higher polarity. RF fractions had a

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composition quite similar to that of DF fractions with an higher amount of the less volatile components. Most of the components identified in the absolute have been previously identified in *P. nigra* bud exudates [8- 10,12-14,31,32,34-35] by different methods of extraction followed by GC-MS analyses with or without derivatization. Some components are only tentatively identified because of both the lack of pure standards and/or of I^T_S of the TMS derivatives that limits their identification to the comparison to mass spectra available from libraries or from the literature. Percent normalization is here applied as a preliminary indication of abundance of identified and unidentified peaks. Unfortunately some phenolic acids present very bad peak shape (e.g. benzoic acid (9)) or are in very low amounts (e.g. *p*-coumaric acid (47)) when analyzed without derivatization that make difficult a correct peak area integration thus affecting the resulting percentages. GC-FID with derivatization produces phenolic acid peaks easy to integrate but interfering with flavonoid measurement (see below). 91 components were characterized by GC-MS with derivatization and 52 by GC-MS without derivatization. On the basis of component relative % abundance, the absolute consists of about 26% of flavonoids, about 22% of phenolic acids and about 14% of phenolic esters. Terpenoids, aromatic alcohols, and fatty acids and esters have to be considered as minor groups each one representing about 2% of the total absolute.

HPLC-PDA-MS analysis

Poplar bud absolute, DF and RF and their sub-fractions were in parallel analyzed by HPLC-PDA-ESI-qMS. Table 2 reports the list of the components identified by HPLC-PDA-ESI-qMS in the total absolute, DF and RF together with their UV absorption maxima and MS data, while Fig. 2 reports the HPLC-PDA chromatogram of the total absolute. VF fraction was not analyzed by HPLC because the volatility of its components made GC the technique of choice for its analysis. The components were located in the chromatograms in function of their elution order thanks to the available standards, the analysis of some of the DF and RF sub-fractions also analyzed by GC-MS and the data reported in literature [36,37]. Mass spectra were acquired in positive and negative ESI ionization full scan, in general providing a protonated [MH]$^+$ or a deprotonated [M–H]$^-$ molecular ions and, in positive mode, a molecular ion adduct [M + Na]$^+$. The molecular mass of an unknown component was retained only when both [MH]$^+$ and [M–H]$^-$ ions were detected. LC-MS data are here fundamental not only for component characterization and location in the chromatogram but also as a complement to confirm GC-MS identification. HPLC results confirmed that *P. nigra* absolute mainly consists of three groups of phenolic compounds (phenolic acids, phenolic esters and flavonoids) as already shown by GC analysis. 30 components were characterized both by HPLC-PDA-MS analysis, two of them not identified by GC-MS with or without derivatization.

Quantitation of the marker components of poplar absolute

On the basis of the above results showing the prominence of the three phenolic fractions and of the availability of standards, fourteen phenolic compounds were selected as markers to characterize the poplar bud absolute and quantified via an external calibration by both GC-SIM-MS and HPLC-PDA to validate the data by two independent chromatographic methodologies. These compounds are listed in Tables 3 and 4 and were chosen also because the absolute qualitative composition is influenced by the procedure adopted for its preparation that limits the flavonoids to those belonging to the subgroups of flavanones, flavones and dihydroflavonols deriving from cinnamoyl-CoA and pinostrobin chalcone without substituents in B ring. Table 3 reports the diagnostic ions selected for GC-SIM-MS quantitation, absorption maxima wavelengths (λ max) adopted for HPLC-PDA

quantitation, together with the calibration ranges, correlation coefficients (r^2) and detection and quantitation limits (LOD and LOQ) of each marker. Table 4 compares the amounts of the 14 markers in the poplar bud absolute under investigation obtained with GC-SIM-MS after derivatization (n = 3) and with HPLC-PDA (n = 3) expressed as mg/100mg of total absolute together with the RSD% between the results. The quantitative results of the main components using the two different methods are comparable for most markers (RSD% < 20) and emphasizes the reliability of both techniques in quantifying the markers of this complex matrix. RSD% values of some flavonoids (in particular pinostrobin, 29,6%, and chrysin, 28,1%) when analyzed by GC-SIM-MS with derivatization and HPLC-PDA above 20% can either be due to possible coelution with other components with both derivatization GC-MS and HPLC-PDA, or to an incomplete derivatization of them (table 1a). The latter explanation was also confirmed by the relatively narrow range of linearity obtained for these compounds , by the detection of partially silylated derivatives and by the fact that their HPLC-PDA-UV quantitative data well agreed with those obtained with GC-SIM-MS without derivatization as it is shown by RSDs% that are always below 20% (pinostrobin (6.1%) and chrysin (18.3%), although these compounds present free hydroxyl(s) in the ring A of the flavonoidic skeleton. The quantitation of components for which standards are not available in HPLC-PDA analysis, was carried out by adopting the calibration curves of available standards belonging to the same chemical groups and with the same chromophores and UV absorption maxima, e.g. the calibration curves of ferulic and caffeic acid can be used to quantify ferulic and caffeic esters. The quantitative analysis of the 14 markers and their derivatives selected with the above approximation afforded to quantify 45% by weight of the absolute.

Conclusions

The proposed strategy has here been shown to be able to characterize the poplar bud absolute because it affords to characterize about one hundred components, the main ones of them belonging to the phenolic groups that account for about 45% of the whole absolute, and to define and quantify fourteen markers representative of about 20% of the sample. These results show how non-targeted and targeted methods can successfully be combined when a complex mixture of vegetable origin has to be studied, and how GC-MS with and without derivatization, HPLC-PDA, and HPLC-MS play a fundamental complementary role in the characterization of complex mixtures consisting of components of different volatility and polarity. As already stressed by other authors [18,36], all techniques equally and sinergically concur to the highly heterogeneous and natural complex mixture characterization, in particular when studies are carried out with conventional instrumentation (GC-FID-MS and HPLC-PDA-qMS). This approach will probably be even more effective by the routine adoption of the recent and more powerful HPLC-MS and GC-MS systems based on high resolution IT or TOF analyzers as such or in a hybrid combination (q-TOF or IT-TOF), that increase the capability of component identification since they can provide molecular formulae and enable to propose hypothesis of structures through dedicated software [38]. However, although very powerful, these instrumentations too are limited by the absolute composition, which mainly consists of isomers or homologous components. These results furthermore confirm the importance of derivatization that makes possible to use GC in the analysis of components poorly compatible with it (e.g. benzoic acid) and to exploit its advantages. Nevertheless, it is evident that the generalized use of derivatization both for identification and quantitation of components in a complex mixture of vegetable origin still requires further developments. Few mass spectra and I^T_S of TMS derivatives are available from the commercial libraries (NIST, etc.), thus making difficult component identification if reference standards are not available. These data

can only be found in dedicated articles [18] or in studies concerning species belonging to the same genus or, as in this case, from products directly derived from the same plant, e.g. poplar based propolis [36,37].

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Figure Captions

Fig. 1 Poplar absolute GC-MS profiles as such (A) and after derivatization (B). Peak numbers are referred to those reported in Table 1

Fig. 2 HPLC-PDA profile of poplar absolute. The chromatogram has been acquired at 280 nm. Peak numbers are referred to those reported in Table 1

Supplementary files Captions

Fig. S1 GC-MS profiles of the molecular distillation fractions without derivatization

Fig. S2 GC-MS profiles of the molecular distillation fractions with derivatization.

File S3 List of the main unidentified compounds in the GC-MS profile with derivatization

File S4 List of the main unidentified compounds in the GC-MS profile without derivatization
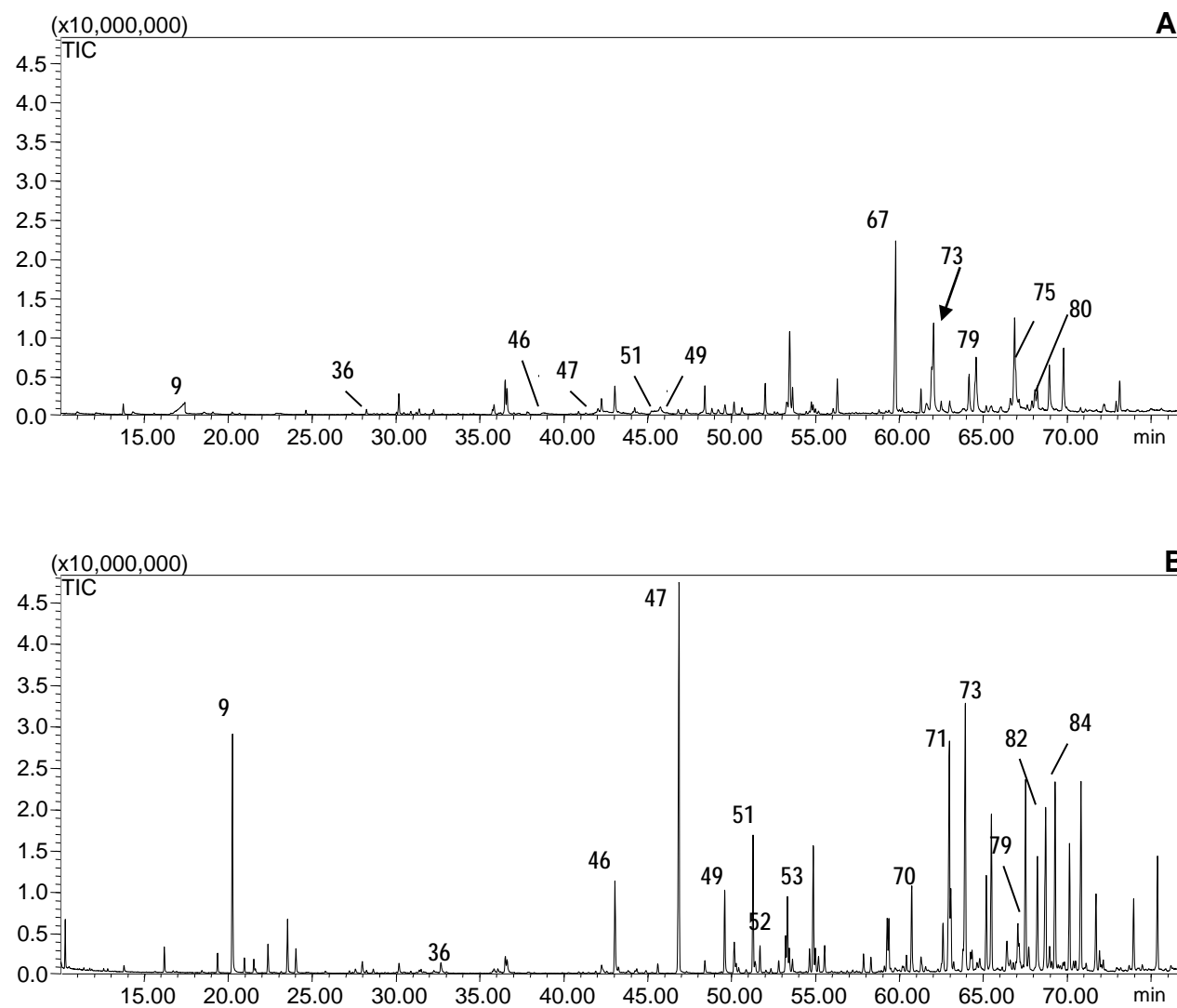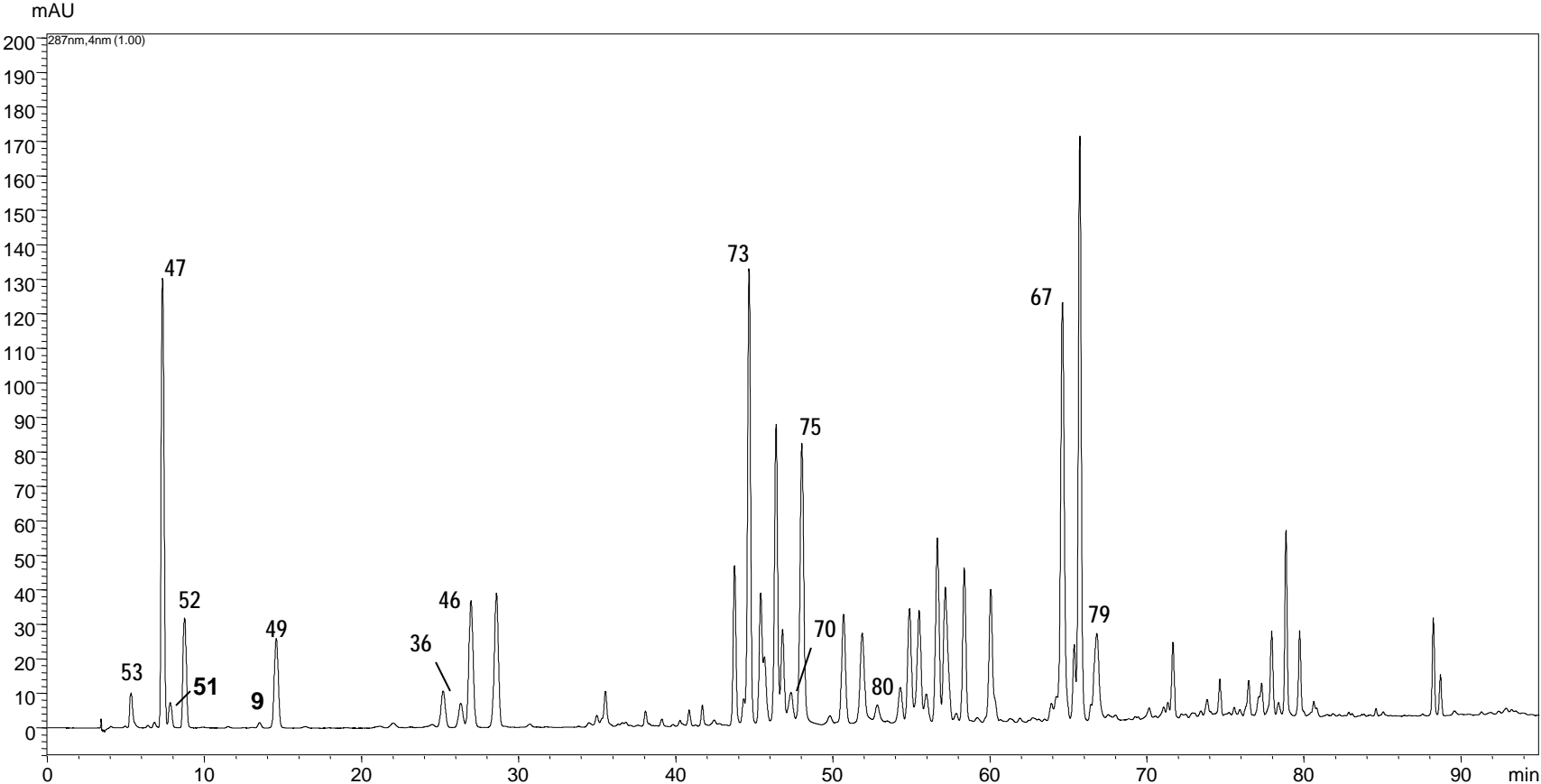
Fig. 1

Fig. 2

Table 1A: Poplar absolute composition obtained through GC-FID-MS analysis after derivatization

ID	Compounds	Fraction	N° TMS groups	I^{T_S} exp	I^{T_S} from literature	%
1	2-Methyl-2-butenoic acid	VF	1	1020	1015[a]	0.6
2	Hexanoic acid	VF	1	1081	1077[b]	0.03
3	Linalool	VF	0	1102	1097[c]	0.1
4	Camphor	VF	0	1146	1146[c]	nd
5	Benzyl alcohol	VF	1	1160	1153[a]	0.3
6	Benzyl acetate	VF	0	1168	1162[c]	nd
7	Ethyl benzoate	VF	0	1173	1163[c]	nd
8	Phenetyl alcohol	VF	1	1230	1223[a]	0.3
9	**Benzoic acid**	VF	1	1251	1244[a]	3.8
10	Phenethyl acetate	VF	0	1260	1258[c]	nd
11	1,2-Cyclohexandiol	VF	0	1267	1262[a]	0.2
12	1,2-Cyclohexandiol	VF	2	1279	1275[a]	0.3
13	p-Vinyl-phenol	VF	1	1281	1281	nd
14	Glycerol	VF	3	1297	1296[a]	0.4
15	Catechol	VF	2	1325	1321[a]	0.9
16	2-(3-phenyl)-propionate	VF	0	1352	1354[c]	nd
17	Hydroquinone	VF	2	1411	1406[b]	0.04
18	*cis-α-Bergamotene*	VF	0	1416	1413[c]	nd
19	β-Caryophyllene	VF	0	1418	1419[c]	nd
20	Hydrocinnamic acid	VF	1	1420	1414[a]	0.1
21	Cinnamyl alcohol	VF	1	1431	1431	0.3
22	*trans-α-Bergamotene*	VF	0	1437	1435[c]	0.1
23	2-Methoxy-4-vinyl phenol		1	1445	1445	nd
24	*o*-Hydroxy-benzyl alcohol	VF	2	1447	1447	0.1
25	α-Humulene	VF	0	1453	1455[c]	nd
26	Alloaromadendrene	VF	0	1460	1461[c]	nd
27	*γ-Curcumene*	VF	0	1481	1483[c]	nd
28	*ar-Curcumene*	VF	0	1484	1481[c]	0.2
29	*1,11-Epoxycalamenene*	VF	0	1491	1490[c]	nd
30	*β-Bisabolene*	VF	0	1510	1506[c]	0.03
31	*Sesquicineole*	VF	0	1514	1516[c]	0.1
32	Salicylic acid	VF	2	1521	1514[b]	0.03
33	*δ-Cadinene*	VF	0	1524	1523[c]	nd
34	*italicene ether*	VF	0	1534	1538[c]	nd
35	Vanillin	VF/DF	1	1540	1533[b]	nd
36	**Cinnamic acid**	VF/DF	1	1548	1544[d]	0.3
37	Nerolidol	VF/DF	0	1567	1563[c]	nd
38	*Eremoligenol*	VF	0	1625	1631[c]	nd
39	*γ-Eudesmol*	VF/DF	0	1631	1630[c]	0.1
40	*4-Hydroxy-benzoic acid*	VF	2	1637	1632[e]	0.1
41	β-Eudesmol	VF/DF	0	1650	1651[c]	0.3
42	*α-Eudesmol*	VF/DF	0	1652	1653[c]	0.3
43	α-Bisabolol	VF	0	1685	1686[c]	nd
44	Vanillic acid	VF/DF	2	1778	1776[b]	0.02
45	*Cryptomeridiol*	VF	0	1810	1814[c]	0.1
46	*p*-Methoxy cinnamic acid	VF/DF	1	1834	1828[d]	1.6
47	*p*-Coumaric acid	DF/RF	1	1951	1943[a]	7
48	Ethyl hexadecanoate	VF/DF/RF	0	1998	1993[c]	0.2
49	**3,4 Dimethoxycinnamic acid**	DF/RF	1	2037	2034[d]	1.4
50	Hexadecanoic acid	VF/DF/RF	1	2055	2050[a]	0.7
51	**Isoferulic acid**	DF/RF	2	2091	2078[d]	2
52	**Ferulic acid**	DF/RF	2	2105	2101[d]	0.4
53	**Caffeic acid**	DF/RF	3	2157	2153[d]	0.5
54	*3-Methyl-3-butenyl-p-coumarate*	DF/RF	1	2161	2159[d]	1.2
55	Ethyl linoleate	VF/DF/RF	0	2162	2162	0.4
56	Ethyl linolenate	VF/DF	0	2169	2169	0.2
57	*2-Methyl-2-butenyl-p-coumarate*	DF/RF	1	2199	2203[d]	0.02
58	*Methyl butenyl-p-coumarate* (t)	DF/RF	1	2205		0.4

ID	Compounds	Fraction	N° TMS groups	IT_S exp	IT_S from literature	%
59	*3-Methyl-2-butenyl-p-coumarate*	DF/RF	1	2214	2218[d]	2.1
60	Linoleic acid	DF	1	2218	2218[e]	0.4
61	Linolenic acid	DF	1	2225	2225[e]	0.3
62	*3-Methyl-3-butenyl-ferulate*	DF	1	2321	2318[d]	0.3
63	*Methyl butenyl (iso)ferulate* (t)	DF		2360		0.05
64	*2-Methyl-2-butenyl isoferulate*	DF	1	2367	2348[d]	0.1
65	*3-Methyl-3-butenyl caffeate*	DF/RF	2?0	2373	2368[d]	0.9
66	*3-Methyl-2-butenyl(iso)ferulate*	DF/RF	1	2376	2373[d]	0.9
67	Pinostrobin	DF/RF	0	2390	2386[d]	n.d.
68	*2-Methyl-2-(iso)butenyl-(iso)ferulate*	DF/RF	1	2396		0.03
69	*2-Methyl-2-butenyl-caffeate*	DF/RF	2	2416	2412[d]	0.3
70	**Dimethyl allyl caffeate**	DF/RF	2	2427	2424[d]	1.6
71	**Pinostrobin**	DF/RF	1	2514		4.9
72	*Benzyl-p-coumarate*	RF	1	2517	2515[e]	1.4
73	**Pinocembrin**	DF/RF	2	2551	2550[d]	5.9
74	*Pinobanksin*	DF/RF	3	2611	2610[d]	3.0
75	Chrisyn	DF/RF	1	2650	2645[d]	0.8
76	*Benzyl isoferulate*	DF/RF	1	2658		0.3
77	*Benzyl ferulate*	DF/RF	1	2679		0.4
78	*Pinobanksin 3-acetate*	DF/RF	2	2695	2695[d]	3.5
79	**Tectochrysin**	DF/RF	1	2704	2700[d]	0.6
80	Galangin	DF/RF	2	2717	2716[d]	n.d.
81	*Benzyl caffeate*	DF/RF		2725	2723[e]	2.7
82	**Chrysin**	DF/RF	2	2746	2742[d]	3.4
83	*Methoxy chrysin (t)*	DF/RF		2757		0.6
84	**Galangin**	DF/RF	3	2770	2767[d]	3.2
85	*Pinobanksin-3- butanoate (t)*	DF/RF	2	2793		0.2
86	2-Phenylethyl caffeate (CAPE)	DF/RF	2	2807	2804[d]	2.5
87	*Cinnamyl p-coumarate*	DF/RF	1	2838		3.9
88	*Pinobankisin-5-methyl ether (t)*	DF/RF	2	2878		1.5
89	*Pinobanksin-3-pentanoate*	DF/RF	2	2887	2884[d]	0.4
90	*Cinnamyl isoferulate*	RF		2977		1.5
91	*Cinnamyl caffeate*	RF		3043		2.3

Compounds whose reference standards are not available are in Italics;
marker components used for the true quantitation are in bold,
n.d.:compounds not detectable in the total chromatogram but present as a trace in the fractions;
(t): tentative in agreement with LC data
[a]:from ref. [12]; [b]:from ref. [16]; [c]:from ref [15]; [d]:from ref [18]; [e]: from ref [17]

Table 1B: Poplar absolute composition obtained through GC-FID-MS analysis without derivatisation

ID	Compounds	Fraction	$I^T{}_S$ exp	$I^T{}_S$ from lierature	%
1	2 Methyl-2-butenoic acid	VF	970	970	nd
5	Benzyl alcohol	VF	1039	1032[c]	0.1
3	Linalool	VF/DF	1101	1097[c]	0.4
8	Phenethyl alcohol	VF	1115	1107[c]	0.2
4	Camphor	VF	1146	1146[c]	0.04
6	Benzyl acetate	VF	1168	1162[c]	nd
7	Ethyl benzoate	VF	1173	1173[c]	nd
9	**Benzoic acid**	VF/DF	1186		2.9
15	Catechol	VF	1204		nd
10	Phenethyl acetate	VF	1260	1258[c]	0.1
21	Cinnamyl alcohol	VF	1308	1304[c]	0.1
23	2-Methoxy-4-vinyl phenol	VF	1315		0.04
20	Hydrocinnamic acid	DF	1346		nd
16	2-(phenyl)-ethyl propionate	VF/DF	1351	1354[c]	0.2
35	Vanillin	VF	1399	1394[c]	nd
18	*cis* α-*Bergamotene*	VF	1415	1413[c]	0.03
19	β-Caryophyllene	VF	1418	1419[c]	nd
36	**Cinnamic acid**	DF	1435		nd
22	*trans*−α-*Bergamotene*	VF	1437	1435[c]	0.3
25	α-Humulene	VF	1453	1455[c]	0.05
27	*γ-Curcumene*	VF	1481	1483[c]	0.1
28	*ar-Curcumene*	VF/DF	1484	1481[c]	0.9
29	*1,11-Epoxycalamenene*	VF	1491	1490[c]	0.1
30	*β-Bisabolene*	VF	1510	1506[c]	0.1
31	*Sesquicineole*	VF/DF	1514	1516[c]	0.3
33	*δ-Cadinene*	VF	1524	1523[c]	0.1
37	Nerolidol	VF/DF	1566	1563[c]	nd
38	*Eremoligenol*	VF	1629	1631[c]	nd
39	*γ-Eudesmol*	VF/DF	1631	1632[c]	0.5
41	β-Eudesmol	VF/DF	1650	1651[c]	1.6
42	*α-Eudesmol*	VF/DF	1653	1654[c]	1.3
43	α-Bisabolol	VF	1687	1686[c]	0.1
46	*p*-**Methoxy cinnamic acid**	DF	1711	1711	0.2
47	*p*-**Coumaric acid**	DF	1797	1797	nd
51	**Isoferulic acid**	DF	1899	1899	0.3
49	**3,4 Dimethoxycinnamic acid**	DF	1916	1916	0.9
48	Ethyl hexadecanoate	VF/DF	1993	1993[c]	0.1
54	*3-Methyl-3-butenyl-p-coumarate*	DF/RF	2116	2110[d]	1.5
60	Linoleic acid	DF	2135	2135	0.1
61	Linolenic acid	DF	2141	2141	0.1
57	*2-Methyl-2-butenyl-p-coumarate*	DF/RF	2160	2154[d]	0.9
59	*3-Methyl-2-butenyl-p-coumarate*	VF/RF	2166	2161[d]	4.8
56	Ethyl linolenate	VF/DF	2171	2171	1.4
62	*3-Methyl-3-butenyl ferulate*	DF	2210	2195[d]	0.6
64	*2-Methyl-2-butenyl ferulate*	DF	2253	2243[d]	0.3
66	*3-Methyl-2-butenyl ferulate*	DF/RF	2261	2257[d]	1.7
67/71	**Pinostrobin**	DF/RF	2391	2386[d]	9.3
73	**Pinocembrin**	DF/RF	2478	2480[d]	6.2
79	**Tectochrysin** + *coelut*	DF/RF	2576	2576[d]	5.0
75/82	**Chrysin** + *coelut*	DF/RF	2669	2660[d]	7.9
80/84	**Galangin**	DF/RF	2719	2718	1.5
88	*Pinobanksin-5-methylether (t)*	DF/RF	2756		3.6

ID number is related to the compounds of Table 1A

compounds whose reference standards are not available are in Italics;

marker compounds used for the true quantitation are in bold;

n.d.:compounds not detectable in the total chromatogram but present as a trace in the fractions;

(t) tentative in agreement with the LC data

[c]: from ref [15]; [d]:from ref [18]

Table 2: Poplar absolute composition obtained through HPLC-PDA-esiMS analysis

ID	Compounds	RT (min)	UV λ max (nm)	M.W.	ESI scan +	ESI scan -
53	Caffeic acid	5.32	325	180		179
47	*p*-Coumaric acid	7.33	309	164	147	163, 119
52	Ferulic acid	7.82	321	194		193
53	Isoferulic acid	8.74	323	194	195	193
9	Benzoic acid	13.07	220	122		121
49	3,4 Dimethoxycinnamic acid	14.57	321	208	209, 191	
88	Pinobanksin-5-methyl-ether[a]	25.196	287	286	287 309 [M+Na]+	285
36	Cinnamic acid	26.315	278	148	149	147
46	*P*-Methoxy-cinnamic acid	26.973	307	178	179	181
74	Pinobanksin[a]	28.59	291	272	273	271
73	Pinocembrin	44.67	290	256	257	255
81	Benzyl caffeate[a]	45.41	329	270	271	269
78	Pinobanksin-3-*O*-acetate[a,]	46.39	293	314	315 273 [M+ H – acetate]+	313 253 [M -acetate – H$_2$O)]+
65/69	Methyl-butenyl caffeate[b]	46.80	326	248	249	247
70	Dimethyl allyl caffeate	47.34	326	248	195	247
75/82	Chrysin	48.03	267/314	254	255	253
86	2-Phenyl-ethyl caffeate (CAPE)[A]	50.69	326	284	285	283
84	Galangin	51.88	265/359	270	271	269
83	Methoxy-chrysi n[a]	54.30	266/341	284	285	283
72	Benzyl *p*-coumarate[a]	54.888	312	254	277 [M+Na]+	253
54/57 /59	Methyl-butenyl-*p*-coumarate[b]	55.501	313	232	233	231
54/57 /59	Methyl-butenyl-*p*-coumarate[b]	56.663	311	232	--	231
	Pinobanksin-3-propionate[a]	57.169	295	328	329	327
67/71	Pinostrobin	64.628	289	270	271	269
85	Pinobanksin-3-butanoate[a]	65.366	293	342	343	341
87	Cinnamyl *p*-coumarate[a]	65.728	313	280	303 [M+Na]+	279
79	Tectochrysin	66.816	267/306	268	269	267
89	Pinobanksin-3-pentanoate[a]	71.665	293	356	357 379 ([M+Na]+	355
	Pinobanksin-3-hexanoate[a]	76.48	292	370	371	369
43	Cinnamyl *p*-methoxy-cinnamate[a]	79.73	278	294	295	293

ID number is related to the compounds of Table 1A
Marker compunds are in bold
[a]: confirmed with ref. [35,37]
[b]: tentative on the basis of UV and MS data and coherence with the GC-MS analysis.

Table 3: Poplar absolute markers and parameters adopted for their quantitation

N°	Compounds	Derivatization GC-SIM-MS						HPLC-DAD-UV					
		IT_S	target ions	r^2	Linearity µg/mL	LOD µg/mL	LOQ µg/mL	R.t. min	λ max	r^2	Linearity µg/mL	LOD µg/mL	LOQ µg/mL
9	Benzoic acid	1251	179, 105, 77	0.999	0.05-0.5	0.008	0.022	13.51	230	0.996	1-50	0.005	0.018
36	Cinnamic acid	1544	205, 131, 161	0.997	0.05-0.5	0.028	0.092	22.23	277	0.999	1-50	0.006	0.019
46	p-Methoxycinnamic acid	1834	161, 235, 250	0.991	0.05-0.5	0.039	0.129	23.21	310	0.994	1-50	0.008	0.028
47	p-Coumaric acid	1951	73, 219, 308	0.997	0.05-0.5	0.004	0.014	9.22	310	0.998	1-50	0.001	0.002
49	3,4 Dimethoxycinnamic acid	2037	191, 265, 280	0.991	0.05-0.5	0.111	0.369	17.55	325	0.999	1-50	0.007	0.023
51	Isoferulic acid	2091	73, 338, 308	0.995	0.05-0.5	0.006	0.022	12.27	325	0.999	1-50	0.002	0.008
52	Ferulic acid	2105	73, 338, 249	0.991	0.05-0.5	0.007	0.022	10.82	325	0.998	1-50	0.003	0.008
53	Caffeic acid	2157	73, 219, 396	0.995	0.05-0.5	0.011	0.038	6.18	325	0.999	1-50	0.002	0.008
70	1,1-Dimethylallyl caffeate	2427	73, 219, 292	0.990	0.05-0.5	0.019	0.064	45.34	325	0.999	1-50	0.025	0.083
71	Pinostrobin	2514	327, 73, 238	0.983	0.1-0.5	0.424	1.415	63.19	289	0.997	1-50	0.029	0.098
73	Pinocembrin	2551	73, 385, 296	0.995	0.1-0.5	0.007	0.022	43.34	290	0.993	1-50	0.014	0.047
79	Tectochrysin	2704	325, 155, 282	0.995	0.3-1.00	1.252	4.172	65.49	267	0.998	1-50	0.008	0.027
82	Chrysin	2746	383, 73, 311	0.991	0.25-0.5	0.028	0.092	46.48	267	0.997	1-50	0.015	0.051
84	Galangin	2770	571, 73, 399	0.996	0.05-0.5	0.012	0.040	50.11	265	0.995	1-50	0.063	0.210

Table 4: Data comparison of poplar bud absolute markers after true quantitation by derivatization-GC-MS and HPLC-PDA

N°	Compounds	True quantitation		
		Der-GC-MS mg/100mg (%)	HPLC-PDA mg/100mg (%)	RSD%
9	Benzoic acid	1.24	1.26	1.2
36	Cinnamic acid	0.23	0.21	6.4
46	*p*-Methoxycinnamic acid	1.30	1.3	0.0
47	*p*-Coumaric acid	1.92	1.8	4.6
49	3,4 Dimethoxycinnamic acid	1.30	1.07	13.5
51	Isoferulic acid	0.99	0.93	4.1
52	Ferulic acid	0.42	0.32	19.0
53	Caffeic acid	0.44	0.34	18.9
70	1,1-Dimethylallyl caffeate	0.92	0.99	5.1
71	Pinostrobin	3.48	5.32	29.6
73	Pinocembrin	2.79	3.64	18.8
79	Tectochrysin	1.87	1.37	21.6
82	Chrysin	2.78	4.15	28.1
84	Galangin	2.24	2.89	17.9