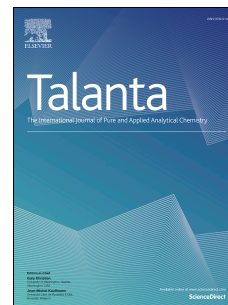


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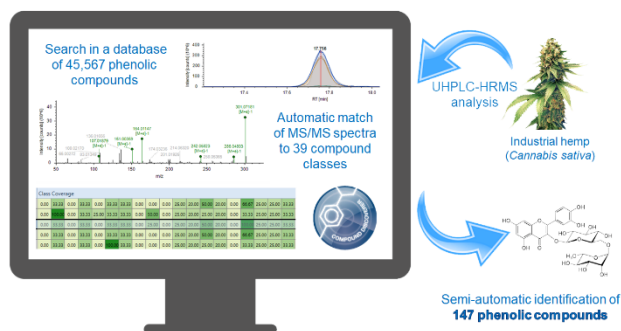
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A new software-assisted analytical workflow based on high-resolution mass spectrometry for the systematic study of phenolic compounds in complex matrices

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Keywords

Cannabis sativa; high resolution mass spectrometry; polyphenols; ultra-high performance liquid chromatography; Compound Discoverer

Abstract

Polyphenols are a broad class of plant secondary metabolites which carry out several biological functions for plant growth and protection and are of great interest as nutraceuticals for their antioxidant properties. However, due to their structural variability and complexity, the mass-spectrometric analysis of polyphenol content in plant matrices is still an issue. In this work, a novel approach for the identification of several classes of polyphenol derivatives based on ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry was developed. First, mass-spectrometric parameters were optimized in order to obtain a large set of diagnostic product ions for their high-confidence identification. The software Compound Discoverer 3.0 was then implemented with a comprehensive database of 45,567 polyphenol derivatives and with mass-spectrometric data for their building blocks, resulting in a specific tool for the semi-automatic identification of flavonoids, anthocyanins, ellagitannins, proanthocyanidins and phenolic acids. The method was then applied to the identification of polyphenols in industrial hemp (*Cannabis sativa*), a matrix whose use is recently spreading for pharmaceutical and nutraceutical purposes, resulting in the identification of 147 compounds belonging to the classes of flavonoids, proanthocyanidins and phenolic acids. The proposed method is applicable to the polyphenol profiling of any plant matrix and it is not dependent on data in the literature for their identification, allowing the discovery of compounds which have been never identified before.

Abbreviations

HRMS: High-resolution mass spectrometry

DDA: Data dependent acquisition

LC-MS: Liquid chromatography – mass spectrometry

HCD: Higher-collisional dissociation

CE: Collision energy

NCE: Normalized collision energy

FWHM: Full width at half maximum

XIC: Extracted ion chromatogram

RDA: Retro Diels-Alder

HRF: Heterocyclic ring fission

QM: Quinone methide

1. Introduction

Phenolic compounds constitute a broad class of naturally occurring phytochemicals structurally characterized by the presence of one (e.g. phenolic acids, phenols) or several phenolic groups (e.g. polyphenols), which are capable of reducing reactive oxygen species. Due to their structural variability, phenolic compounds serve more than one function both for plant growth and resistance, and in response to environmental constraints[1]. Phenolic acid derivatives, for instance, have been proven to play a key role in secondary cell wall thickening, thus conferring plants a greater drought tolerance[2]. Moreover, light-absorbing polyphenol biosynthesis increases when plants are exposed to high sunlight to protect the leaves from UV-B radiation damaging effects[3,4]. Finally, polyphenols possessing catechol groups can efficiently chelate metal ions, which generate extremely dangerous free radicals due to Fenton reactions in presence of H_2O_2 [5].

As a result of their abundance in vegetable matrices, phenolic compounds represent an abundant class of micronutrients in human diet, possibly playing important biological roles for human health.[6,7] Indeed, oxidative damage to cell components, DNA, proteins and lipids, contributes to the pathogenesis of degenerative diseases such as cancers, cardiovascular diseases, osteoporosis and neurodegenerative diseases. Chain oxidation reactions, which are responsible for the deterioration of cellular components, can be dwindled and inactivated by antioxidants present in food, such as polyphenols, as they are capable of subtracting one electron from free radicals, forming relatively stable phenoxyl radical species[8].

Due to the relationship between phenol compound structures and antioxidant activity[9], the development of an analytical method for their identification is essential for the evaluation of plants health effect. Phenolic compounds are generally subdivided in two main classes, flavonoids, such as flavones, flavonols, flavonones, flavanols, isoflavones and anthocyanins, and phenolic acids, such as hydroxybenzoic and hydroxycinnamic acids. Furthermore, polymeric phenolics, termed tannins, are also normally grouped in two classes, namely hydrolysable tannins (ellagitannins) and non-

hydrolysable tannins (proanthocyanidins). Moreover, phenolics frequently occur as glycosides, methylated and acylated derivatives[9]. The extreme complexity of phenol compound class in terms of structural and chemical properties renders their comprehensive profiling and identification an intricate issue. High performance liquid chromatography coupled to high resolution mass spectrometry (HRMS) in data dependent acquisition mode (DDA) is the most preferred analytical technique for untargeted polyphenol profiling since it allows the chromatographic separation of complex mixtures and the simultaneous acquisition of HRMS and MS² data[10–14]. Polyphenol identification has been usually achieved by an intense manual investigation of MS spectra for searching the precursor ions, and MS² spectra for matching the fragmentation patterns with those reported in the literature, since currently available databases are often incomplete. Thus far, polyphenol identifications in plant matrices have been usually limited to compounds which were already reported in the literature. Recently, a structural feature guided “fishing” strategy was proposed by Liu et al.[11] in order to bypass the aforementioned limitations, with possible search for previously unknown compounds.

In this paper, a novel analysis workflow for polyphenol identification has been implemented on Compound Discoverer 3.0, a software dedicated to the identification of small molecules based on HRMS analysis. The main purposes of the work were: i) enlarging the number of identifiable unknown polyphenols; ii) reducing compound manual validation; and iii) reducing false positive identifications.

Firstly, for the comprehensive profiling of polyphenols, a database with the molecular weights and formulas of 45,567 polyphenol derivatives with sugars and organic acids was compiled and implemented on Compound Discoverer 3.0. Secondly, the MS parameters for higher-collisional dissociation (HCD) were investigated to obtain high-quality MS² spectra for isomer discrimination. Moreover, a deep and detailed study of the fragmentations of non-conjugated phenolics was performed and the patterns were implemented on the software for compound classes scoring based on MS² spectra matching.

In order to explore the potential of the processing workflow, the method was applied to the polyphenol profiling of industrial hemp flowers from the *Kompolti* variety, which is extensively cultivated in Europe and provides raw material for the most diverse uses, from fibers and fabrics to nutraceutical applications. The interest in *Cannabis sativa* has recently increased as a result of the international trends towards decriminalization or legalization of hemp for therapeutic and recreational uses. In the pharmaceutical field, two phenotypes of hemp are distinguished, according to the content in the psychoactive principle Δ^9 -tetrahydrocannabinol (Δ^9 -THC)[15]. The pharmaceutical interest in this plant has been traditionally addressed to drug-type cannabis, thanks to its several therapeutic applications[16], whereas industrial hemp is still under-employed for pharmaceutical and nutraceutical purposes. Among the several classes of bioactive compounds present in industrial hemp, non-psychoactive cannabinoids, such as cannabidiol and cannabigerol, in fact, represent the sole class of phytochemicals that has been widely characterized[17]. Hence, a deep and detailed profiling of the polyphenol content of industrial hemp is of great interest for the aforementioned applications in therapeutic and nutraceutical fields.

2. Materials and methods

2.1 Chemicals and reagents

The industrial hemp analyzed is of the *Kompolti* variety, cultivated in Glaginzano Terme (Padua, Italy). Flowers were gathered at full-flowering stage in the middle of May 2019. The flowers were mashed, freeze-dried by a Heto PowerDry LL1500 (Thermo Fisher), finely ground in a mortar and stored at -20 °C until use. Optima® LC-MS grade water, methanol (MeOH) and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Hexane, acetone, acetic acid and formic acid were purchased from Merck (Kenilworth, New Jersey, USA). Apigenin, apigenin 7-*O*-glucoside, biochanin A, 3-caffeoylquinic acid, caffeic acid, catechin gallate, cyanidin,

diosmetin, epicatechin, epicatechin gallate, eriodyctiol, ferulic acid, formononetin, genistein, genistein, hesperetin, hesperidin, kaempferol, kuromanin, luteolin, malvidin 3-*O*-galactoside, myricetin, naringenin, *p*-coumaric acid, peonidin 3-*O*-glucoside, procyanidin B1, procyanidin B2, resveratrol, rutin, quercetin, quercetin 3-*O*-glucoside and taxifolin analytical standards were purchased from Sigma-Aldrich now Merck. Catechin, isorhamnetin, luteolin 7-*O*-glucoside, malvidin chloride, morin and phloretin analytical standards were purchased from Extrasynthese (Lyon, France). A standard mixture of the aforementioned analytical standards was prepared at 0.5 $\mu\text{g mL}^{-1}$ in $\text{H}_2\text{O}/\text{MeOH}$ 90:10 (v/v), aliquoted and stored at -20 °C for further use.

2.2 Sample preparation

Phenolic compounds were extracted following the protocol of Pellati et al.[17] with some modifications. Briefly, 0.25 g of freeze-dried hemp sample were extracted with 10 mL of *n*-hexane for 15 min under sonication in an ice bath, to remove non-psychoactive cannabinoids. Sample was centrifuged for 10 min at $2000 \times g$ and the supernatant was discarded. The procedure was repeated twice more. The residue was dried under nitrogen flow and, subsequently, it was extracted with 10 mL $\text{CH}_3\text{COCH}_3/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$ (70:29.5:0.5, v/v/v). The extract was sonicated for 15 min in an ice bath and then centrifuged for 10 min at $2000 \times g$. The supernatant was collected and the procedure was repeated once. The supernatants were mixed and concentrated to 4.5 mL using a Speed-Vac SC 250 Express (Thermo 164 avant, Holbrook, NY, USA). Then, 500 μL of MeOH were added to the sample and the final extract solution ($\text{H}_2\text{O}/\text{MeOH}$, 90:10 v/v) was filtered through a 13-mm Acrodisc Syringe filter with a 0.2 μm GH Polypro membrane (Pall, Ann Arbor, MI, USA). Finally, the extract was aliquoted and stored at -20 °C for further analysis.

2.3 UHPLC-ESI-MS² analysis

A Vanquish binary pump H (Thermo Fisher Scientific, Bremen, Germany), equipped with thermostated autosampler and column compartment, was used for polyphenol chromatographic separation on a Kinetex core-shell C₁₈ column (100 mm × 2.1 mm i.d.) with particle size of 2.6 μm (Phenomenex, Torrance, CA, USA) at 40 °C and with a flow-rate of 600 μL min⁻¹. The elution gradient and mobile phases were optimized in a previous work[12]. The mobile phases consisted in H₂O/HCOOH (99.9:0.1, v/v; phase A) and ACN/HCOOH (99.9:0.1, v/v; phase B). The gradient was as follows: 5-15% phase B in 10 min, 15-35% phase B in 15 min, 35-50% phase B in 5 min; at the end of the gradient a washing step at 95% phase B for 5 min and a re-equilibration step at 5% phase B were performed.

The chromatographic system was coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) with a heated ESI source. The ESI source parameters were set as reported in our previous work[18]. Spray voltage was set at 3500 V in positive mode and 2500 V in negative mode; capillary temperature was 275 °C in positive mode and 350 °C in negative mode; auxiliary gas was set at 15 arbitrary units (a.u.); sweep gas was set at 2.25 a.u. in positive mode and 3 a.u. in negative mode; auxiliary gas heater temperature was 450 °C in positive mode and 300 °C in negative mode and S-lens RF level was 50%.

For both low and high-molecular weight polyphenols, detection was conducted in TOP 5 DDA acquisition mode, consisting in a first full-scan acquisition, followed by the fragmentation of the five most intense ions detected in full-scan mode. DDA methods are particularly suitable with Orbitrap-based system, as they provide relatively slow acquisition; the selection of the most intense ions assures higher-quality and clearer fragmentation spectra than other techniques, like all-ion fragmentation (AIF) and data-independent acquisition mode (DIA). An inclusion list deriving from a customized polyphenol database was added to the TOP 5 DDA method for MS² acquisition (see section 2.4). Also, an exclusion list containing the most intense ions detected in a blank sample, consisting of H₂O/MeOH (90:10, v/v), was added to the mass-spectrometric method.

For low-molecular weight polyphenol analysis (flavonoids and phenolic acids) and for high-molecular weight polyphenol analysis (tannins), MS data were acquired in the range 150-1000 m/z and 300-2000 m/z , respectively, with a resolution (full width at half maximum, FWHM, at m/z 200) of 70,000. In full scan mode, the automatic gain control (AGC) target value was 200,000 and the maximum ion injection time was 100 ms. The isolation window width was 2 m/z . MS² fragmentation was performed with a resolution (FWHM, at m/z 200) of 35,000 with AGC target value set at 100,000 and dynamic exclusion set to 3 s. A stepped collision energy (CE) fragmentation was achieved in the HCD cell at three values of normalized collision energy (NCE), namely, 20-50-80 NCE in positive mode and 20-40-60 NCE in negative mode. All samples were run in triplicate followed by the injection of the standard mix and a blank sample of H₂O/MeOH (90:10, v/v). The injection volume was 10 μ L.

2.4 Polyphenol database compilation

To the extent of performing the untargeted analysis focused on phenolic compounds, a customized database containing exact masses of several classes was implemented in the Compound Discoverer software (v. 3.0, Thermo, Waltham, USA). The database included flavonoids, phenolic acids, phenolic aldehydes, ellagitannins and proanthocyanidins. The exact masses and chemical formulas of some selected primary compounds were combined using Excel 2016 (Microsoft), by taking into account the mass loss due to the chemical bond formed.

As for the flavonoids, phenolic acids and phenolic aldehydes, the aglycone and glycosylated forms were included, along with their esters with common acids. The “bricks” used to build this part of the database were flavones, flavonols, flavanols, flavanones, flavanonols, isoflavones, anthocyanins, cannflavins, hydroxybenzoic acids, hydroxycinnamic acids and some other related phenolic compounds. The complete list is shown in Table S1. The aglycones and phenolic acids were combined with one, two or three sugar moieties (pentose, hexose, deoxypentose, deoxyhexose,

glucuronic acid); several acids (including phenolic acids thereof) bound directly to the aglycone or to the glycosylated compound through ester bond were considered as well. A scheme of all considered combinations for flavonoids and phenolic acids is provided in Figure 1.

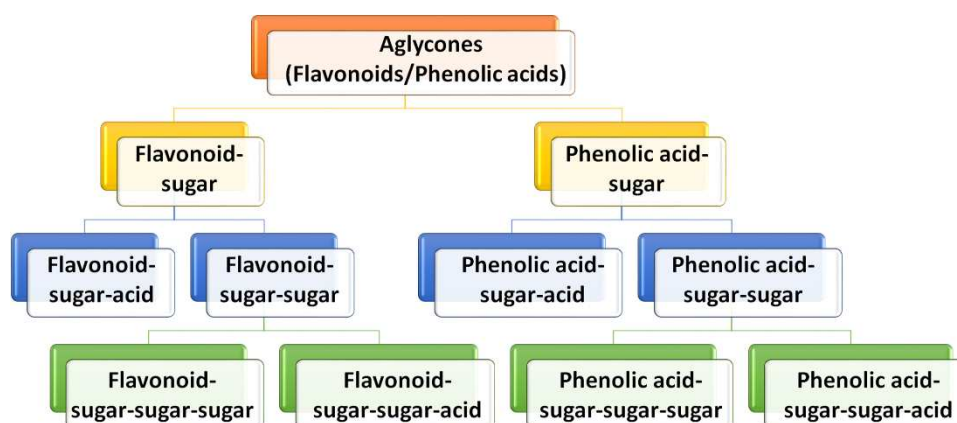


Figure 1. Combination of flavonoid and phenolic acid derivatives generated for the polyphenol database

As far as ellagitannins are concerned, the basic core of the molecule, namely pentose, hexose, deoxyhexose or quinic acid, was combined with up to five moieties of gallic acid, ellagic acid, methyl-ellagic acid and hexahydroxydiphenic acid (considered as two moieties); moreover, the following ellagitannins of known molecular mass were included: pedunculagin, punicalagin, casuarictin, casuarinin, corilagin, geraniin, nupharin A, sanguin H-6, sanguin H-10, stenophyllanin, strictinin, terflavin B, potentillin, sanguisorbic acid dilactone, valoneic acid, castalin and castalagin. Finally, exact masses of proanthocyanidins were obtained by combining two to five afzelechin, catechin and gallocatechin entities; the gallate esters of these oligomers were considered as well.

A total of 45,567 exact masses were generated and used as a customized database for automatic search of adducts in the raw data files.

2.5 Data analysis and polyphenol validation

For each sample, raw data obtained from three consecutive injections and from the blank sample were processed by Compound Discoverer using a method described as follows. The method is based on a block diagram with a series of steps that are consequentially performed (figure S1). After raw data files are imported and labeled (blank or sample), spectra are selected in the first processing block, while in the second one alignment is performed based on adaptive curve and with a maximum retention time shift of 0.2 min. The third block is “detect compound” tool, in which precursor ions are extracted according to the selected possible adducts. Mass tolerance is set at 5 ppm and maximum element counts was C100 H150 O50 Cl N Na, considering possible sodium, ammonium and chloride adducts. The “group compound” tool performs adduct and isotopic peak grouping with mass tolerance at 5 ppm and RT tolerance at 0.2 min. Fill gaps tool is a crucial step for automatically correcting possible peak detection or alignment errors by searching for missing peaks and, eventually, background compounds are removed if the sample to blank area ratio is less than 5. For both low- and high-molecular weight polyphenols raw data processing, the databases generated as described in section 2.4 and complete of IDs, masses and molecular formulas, were implemented in the *mass list* feature for the automatic matching of extracted m/z ratios. Moreover, detailed HCD fragmentation spectra for flavonoids and phenolic acids were implemented in *compound class scoring* section for automatic MS² spectra matching, and aglycone m/z ratios were added to *mass trace* feature to detect in-source fragments of conjugated phenolics in the extracted-ion chromatogram (XIC). Finally, parameters for *predict composition* tool were adapted to polyphenol analysis with a maximum element counts of C100 H150 O50, in order to automatically filter features possessing other elements in their structures, such as nitrogen, sulfur or phosphorus. Extracted masses from the chromatograms were filtered to remove background compounds found in the blank sample, compounds whose m/z ratios could not derive from masses present in the databases and those which were not fragmented. Furthermore, for flavonoid and phenolic acid data

processing, compounds whose compound class scores were 0% for all compound classes, were also filtered out. Finally, filtered compounds were manually validated by matching fragmentation spectra to those of available standards or to spectra reported in the literature. When data were lacking, phenolic compounds were tentatively identified according to the characteristic fragmentation spectra. Detailed Compound Discoverer workflows and parameters are available in Figure S1 and Table S2.

3. Results and discussion

3.1 Data processing workflow on Compound Discoverer

The Compound Discoverer software allows performing small molecule identification based on exact masses and fragmentation spectra. First, an appropriate database of molecular masses must be selected, by choosing either among the ones provided by the vendor (e.g. mzCloud) or customized mass lists. In our case, to focus the identification on phenolic compounds, a database, divided in three parts, was compiled and implemented in the software. The three separated parts included 23,596 free and conjugated flavonoids, 19,305 free and conjugated phenolic acids and 2,645 proanthocyanidins and ellagitannins, respectively. The selection of the compounds used to build the database was based on the study of the most common phenolic compounds identified in vegetable matrices[12,19–21]. Free flavonoids and phenolic acids, termed aglycones, were included, as well as glycosylated forms with one, two or three sugar moieties. Glycosides are usually found at higher concentration in plants and fruits compared to the free forms, due to their greater polarity and consequent solubility in water. A maximum number of three sugar moieties was considered to build the database, since the identification of larger glycosides in plants is unlikely[20,22]. In addition, the esterification of a hydroxyl group of the aglycone or the bound sugar with aliphatic or aromatic acids was considered; nevertheless, we expected a scarce probability of identifying these species,

due to the lower hydrophilicity of these conjugates and consequently lower concentrations[23]. The use of such wide databases allows discovering polyphenol derivatives which were never identified before, since it is independent on data in the literature.

Differently from other software for unknown identification (e.g. the freeware MZMine 2)[24], Compound Discoverer requires databases of molecular masses, instead of m/z ratios. The possible adducts generated both in positive and negative ionization mode are then automatically computed in the processing workflow. For this reason, anthocyanins, which possess a positive charge formally located on the heterocyclic oxygen, was included in the database with a fictitious mass, namely the real molecular mass subtracted of the proton mass (1.007276 u); this ploy allowed to assign correctly this class of molecules, which do not form proton adducts.

Compound identification was achieved thanks to two main matches performed within the software processing workflow. In fact, the identification workflow on Compound Discoverer was implemented by embedding molecular exact masses of precursor ions, information on class-specific product ions searched in the MS^2 spectra and aglycone precursor ions tracing in the chromatogram, to obtain precise information on in-source fragments. By including a list of characteristic fragment ions, we enabled the “compound class scoring” option, which matches each identified feature with the species included in the “compound class list”, by assigning a percentage score.

By applying these criteria, a large screening of the identified features was accomplished, by selecting only the ones possessing both the exact mass match and a compound class scoring different from 0. This automatic screening was followed by the study and validation of the MS^2 spectra, to assign a confidence level to each identification.

3.2 Optimization of MS^2 fragmentation parameters

Although conjugated and non-conjugated polyphenol MS^2 spectra have been widely studied and the fragmentation mechanisms they undergo are well-known[25–27], the structural complexity of those

compounds still causes several issues in their MS²-based identification. Aglycones present, in fact, very stable aromatic structures, which require high CE to be properly fragmented. At the same time, conjugated polyphenols possess very weak acetal bonds with sugars, which undergo extremely rapid cleavage when subject to HCD fragmentation. With the purpose of obtaining as many as possible diagnostic fragments, four different NCE methods have been tested on the standard mixture and the hemp flower extracts both in positive and in negative polarity: single step NCE 40 (method *a*), two-step NCE 40-80 (method *b*), three-step NCE 20-40-60 (method *c*) and three-step NCE 20-50-80 (method *d*). Recently, a stepped CE method has been proposed for glycopeptide fragmentation and identification, since it allows obtaining information about both the sugar moiety and the amino acid chain[28]. NCE is sometimes preferred to conventional non-normalized CE because automatically compensates for the mass dependency, since the CE needed to achieve optimum fragmentation efficiency has been shown to follow a linear correlation with m/z . Since polyphenols possess very diverse m/z ratios (150-2000 m/z), NCE is far more suitable for obtaining comparable MS² spectra of oligomers with the same repeating units but variable molecular weights. Figure 2 shows the MS² spectrum of a conjugated flavonoid found in industrial hemp extract that has been tentatively identified as quercetin *O*-pentoside *O*-hexoside.

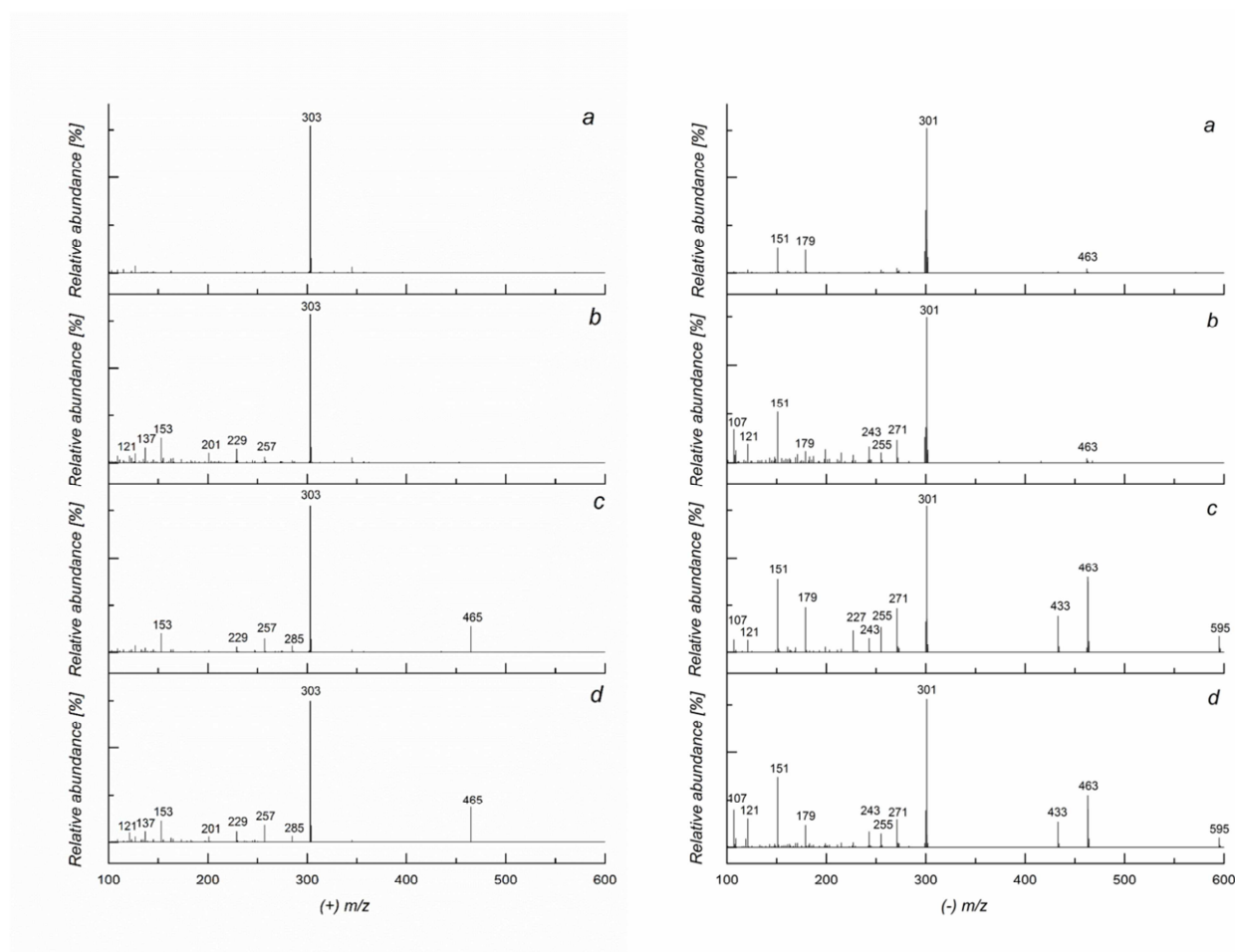


Figure 2: MS² spectra of quercetin O-pentoside O-hexoside obtained with methods *a-d* in positive and negative mode. Fragments in the *m/z* 400-600 range derive from the loss of one of the sugar, those in the *m/z* 200-300 range are generated by neutral losses of small molecules and ions that possess *m/z* below 200 derive from RDA ring openings and heterocyclic ring fissions.

Single step fragmentation at NCE 40 (method *a*) did not provide any information for the sugar moiety and showed a rather scarce fragmentation of the aglycone, especially in positive mode. Method *b* assured a greater fragmentation of the aglycone thanks to the second step at NCE 80, which led to product ions deriving from neutral losses of small molecules and from retro Diels-Alder (RDA) ring opening on the flavonoid skeleton. A low-energy step was consequently added for a milder fragmentation of the acetal bonds between the aglycones and the sugars. Method *c* and *d* consisted in three-step NCE approaches, mediating between low- and high- energy fragmentation

in order to cover all the typical fragmentation pathways. In particular, method *d* (NCE 20-50-80) was chosen for positive ionization mode, since fragments deriving from RDA ring openings and heterocyclic ring fission (HRF) were obtained only with the NCE 80 step, whereas method *c* (NCE 20-40-60) was found to be more suitable for negative mode as it minimized noise and undiagnostic and undesired peaks.

MS² spectra of available standard flavonoids and phenolic acids were recorded with the selected methods and the most intense and characteristic product ions were implemented on Compound Discoverer as compound class scoring features for both polarities. For aglycones whose standards were not available, fragments were chosen according to spectra available in the literature or in MzCloud, a database dedicated to MS spectra recorded with HCD technique. It is worth mentioning that, for all methods tested, negative ionization mode has shown a higher fragmentation efficiency, providing richer and more indicative spectra for the correct identification of isomers. A detailed summary of the available standards and the MS² spectra implemented on Compound Discoverer are available in the Supporting Information (Tables S3-S13).

It must be noted that, although the logic of the customized data analysis approach is extensible to other structure-related classes of molecules or to other mass-spectrometric systems and data analysis programs, the optimum mass-spectrometric parameters must be carefully studied. For polyphenol identification, however, stepped-NCE methods appear to be promising regardless of the instrumentation and the software used, as they furnish a large set of diagnostic product ions.

3.3 Polyphenol identification

Based on the previous evaluation, the best MS² fragmentation methods were chosen. However, due to the structural inconsistency among polyphenols, other parameters were differently set up for the proper HPLC-HRMS analysis of the different classes. Flavonoids and polyphenols are usually present as glycoconjugates to different sugars or sugar derivatives, such as hexoses, deoxyhexoses,

pentoses and glucuronic acid. Moreover, sugars might be acylated to aliphatic and aromatic acids. The general trait of glycoconjugate mass spectra consists of ion signals corresponding both to the protonated or deprotonated glycosylated molecules and the aglycones, which are spontaneously generated by in-source fragmentation during the ionization process. Because of this phenomenon, for the analysis of glycoconjugates, acquisition in the m/z 150-1200 range was performed with a three-stepped NCE of 20-50-80 and 20-40-60 for positive and negative mode, respectively. Two distinct chromatographic runs for each polarity were preferred to polarity switching mode, as it allows obtaining a larger number of data points per peak. For high-molecular weight polyphenols, a suspect screening approach was chosen, since databases for those classes could be used as inclusion lists for the mass-spectrometric method. Top 5 DDA mode is widely used for untargeted analysis with orbitrap-based instrumentation as it allows high quality MS^2 spectra for the five most intense ions for each scan in full-scan mode. However, due to the complexity of phytochemical extracts, low abundance compounds might not be fragmented as many compounds at higher concentrations or ionization efficiency might elute at the same time. As polymeric polyphenols elute in the richest section of the chromatogram (0-10 min), the suspect screening approach ensures those compounds to be fragmented by implementing on the mass-spectrometric method an inclusion list of the m/z ratios, limiting MS^2 fragmentation to ions whose masses are in the list. Acquisition in the m/z 300-2000 range was similarly performed in both polarities with the same NCE methods used for low molecular weight polyphenols. The definitive workflow was firstly applied to the standard mixture analysis, resulting in the correct identification of all the analytes. Final spectra acquisition allowed the identification and tentative identification of 21 and 126 compounds in hemp extract, respectively, including flavones, flavonols, flavanols, flavanonols, hydroxybenzoic acids, hydroxycinnamic acids and proanthocyanidins. Identification of phenolic compounds was based on retention time, accurate mass measurement and MS^2 spectra both for compounds whose standards were available and for those that were identified by match with literature data[10–12,17,25–27,29]. The identification data for the tentatively identified compounds are discussed in the following

sections and summarized in Tables S14, S15 and S16 with the related confidence level according to Schymanski et al.[30]. In particular, level 1 refers to compound identified by match of the aforementioned parameters to those of available standards, level 2a refers to compounds tentatively identified by matching MS² spectra to literature data or online spectral databases, whereas level 2b refers to those tentatively identified by study of diagnostic fragments in MS² spectra but not supported by data available in the literature.

3.3.1 Interpretation of flavonoid spectra

Thirty three analytical standard compounds belonging to various subclasses of flavonoids were available to assist the identification. Retention times of the standards were used to confirm the identity of both aglycones and their conjugates, since the latter are supposed to be eluted earlier from the C₁₈ column due to the polar nature of sugars. Retention times and MS² spectra of standards are summarized in Tables S3-S13. MS² spectra were acquired with three-step NCE methods as previously described, furnishing rich fragmentation patterns. Nomenclature of product ions deriving from flavonoid fragmentation is summarized in Figure 3, while detailed data for identified flavonoids are reported in Table S14.

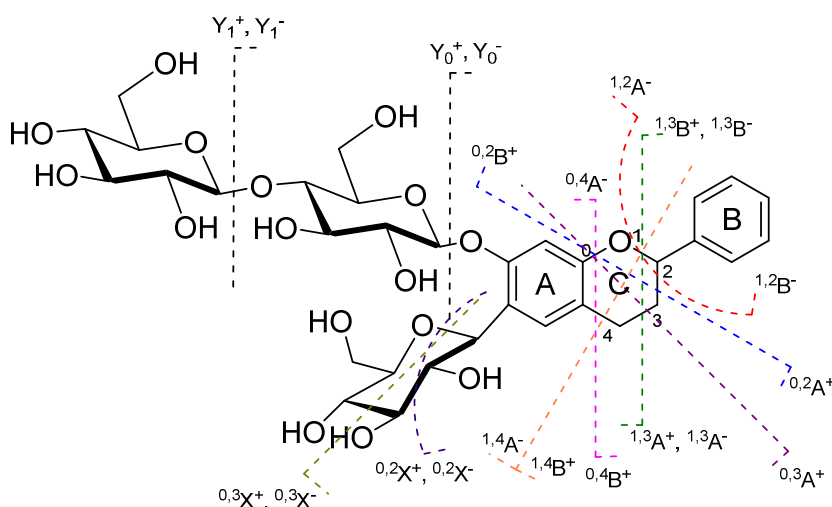


Figure 3. Nomenclature of MS² fragments obtained from flavonoids

O-glycosylated flavonoids, which produce Y fragments deriving from the loss of the sugar moiety, can be easily distinguished from C-glycosylated flavonoids, which generate ions from the internal cleavage of the carbohydrate, named X ions. Compounds 6, 8 and 21 presented, in fact, typical product ions generated by neutral losses of 90.0317 ($C_3H_6O_3$) and 120.0423 ($C_4H_8O_4$) u, indicating the presence of C-linked sugars, and have been tentatively identified as orientin, isoorientin (luteolin C-hexoside) and vitexin (apigenin C-hexoside), respectively. Orientin and isoorientin have been distinguished by the relative ratio between $^{0,3}X^-$ and $^{0,2}X^-$ ions, which has been found to be higher for isoorientin (6-C-glucoside) than orientin (8-C-glucoside)[31]. Compounds 10-13 have been tentatively identified as O-glycosyl-C-glycosyl flavones, since they sequentially produced Y and X ions. Moreover, fragments deriving from the sugar moiety cleavage have been used to speculate the linkage of those on the aglycones. If more than one sugar is present, fragments deriving from the loss of one of them have been used to distinguish the case of two sugars linked together, which produce only one Y_1 ion, from those which were individually linked to the flavonoid, which produce two. For example, compound 19 has been tentatively identified as quercetin O-hexoside O-pentoside, since it produced fragments at m/z 463.0882 (quercetin O-hexoside) and m/z 433.0776 (quercetin O-pentoside), while compounds 24 and 25 were both tentatively identified as quercetin O-deoxyhexosyl hexoside, since they only generated one Y_1 fragment due the loss of the deoxyhexose.

Due to their rather diversified structures, flavonoids are usually arranged in subclasses, whose structural variability generates peculiar fragments due to both neutral losses of small molecules (H_2O , CO, CO_2), to RDA C-ring openings and heterocyclic ring fissions as previously stated. Compound 85 has been tentatively identified as acacetin, since its MS^2 pattern resembled that of its homologous methylated flavone diosmetin more than that of its common isomer biochanin A, with the notable absence of the fragment $^{0,3}B^-$, which is typical of isoflavones (Figure 4). However,

isomers belonging to the same subclass, such as chrysoeriol and diosmetin (compounds 80 and 82), might present identical fragmentations, denying the precise identification of their conjugates.

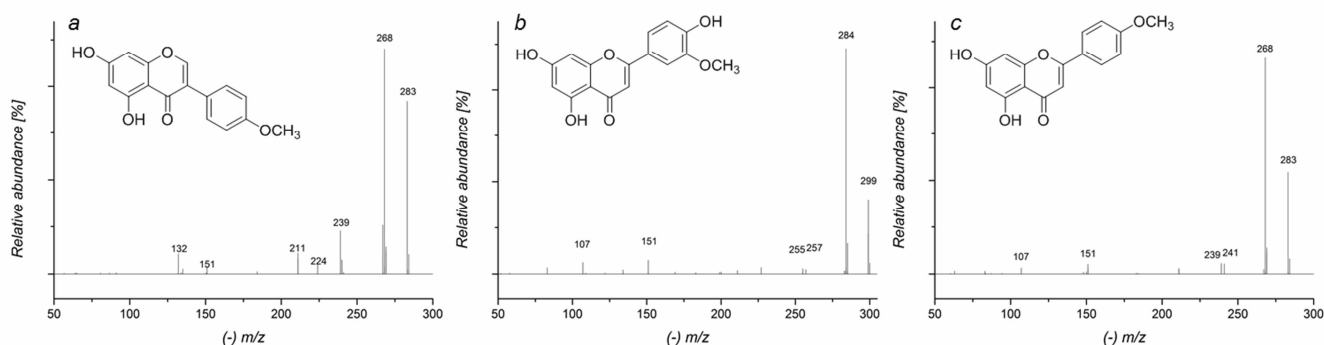


Figure 4. Comparison of the MS² spectra of standard biochanin A (a) and diosmetin (b) to compound c, tentatively identified as acacetin

Finally, compounds 86-88 have been tentatively identified as cannflavins, prenylated flavones unique to cannabis, since they present a typical fragment at m/z 313.0711 in positive mode due to the loss of the prenyl moiety, which undergo further fragmentation with neutral losses of 15 and 28 u.

3.3.2 Interpretation of proanthocyanidin spectra

Proanthocyanidins are non-hydrolysable polymers of flavanols, mainly afzelechin, catechin and gallocatechin, which are distinguished in two subclasses according to their linkage. In particular, A-type proanthocyanidins present a linkage of position 7 and 8 of ring A of the terminal unit to position 2 and 4 of the extension unit; whereas the more common B-type proanthocyanidins are connected from position 8 of the terminal unit to position 4 of the extension unit. Due to the chiral nature of flavanols, which present two stereocenters in their structure, and the chiral nature of both A- and B-type linkage, a large number of proanthocyanidin stereoisomers are expected. The

simplest B-type proanthocyanidin, which is B-type procyanidin dimer (cat-cat), presents 32 stereoisomer, a number that rapidly grow for superior oligomers. However, since those isomers differ only for their chiral nature and possess the same structural linkage, they all present the same fragmentation patterns.

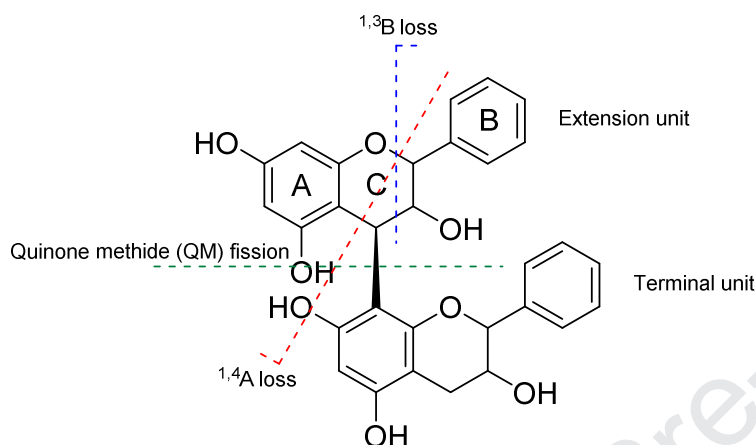


Figure 5. B-type proanthocyanidins fragmentation pathways

The fragmentation pathways of B-type proanthocyanidins generally consist in quinone methide (QM) fissions, RDA ring openings and heterocyclic ring fissions. In particular, QM fissions produce $[M_T-H]^-$ and $[M_E-3H]^-$ ions in negative polarity and $[M_T+H]^+$ and $[M_E-H]^+$ in positive polarity, which can help distinguish the flavanol monomers and their order in the polymeric structure. RDA C-ring opening produces $^{1,3}B$ losses (m/z 136.0524, 152.0473 and 168.0423 for afzelechin, catechin and gallocatechin respectively), which also indicate the nature of the monomers, while HRF $^{1,4}A$ losses produce $[M-126.0317]$ confirming peaks (Figure 5).

Compounds 91, 93, 94, 98, 99 and 102 were all tentatively identified as B-type procyanidin dimers since they produced HRF ions with loss of 126.0317 u, RDA ions with loss of 152.0473 u, which are typical of catechins and QM fission ions at m/z 289.0718 and 287.0561, confirming the presence of two catechins linked together. Similarly, compounds 92, 95-97 and 101 were identified as B-type

procyanidin trimers. Those compounds produced, in fact, m/z 577.1334 and 287.0561 ions as well as m/z 575.1216 and 289.0718 ions, indicating QM fissions between an extension catechin unit and a terminal catechin dimer and between a terminal catechin unit and an extension catechin dimer, respectively. Complete data for identified proanthocyanidins are reported in Table S15.

3.3.3 Interpretation of phenolic acid spectra

Phenolic acids are a wide class of phenolic compounds that includes mainly hydroxylated derivatives of benzoic and cinnamic acid. Similarly to flavonoids, hydroxybenzoic and hydroxycinnamic acids are often conjugated to sugars and other aliphatic or aromatic acids, which increase their polar nature improving their solubility in water. A total of 3 and 42 phenolic acid derivatives were identified and tentatively identified in hemp flower, belonging to the classes of hydroxybenzoic acid glycosides, hydroxycinnamic acid glycosides and hydroxycinnamic acid esters to quinic and shikimic acid. Due to the extremely compact structures of phenolic acid aglycones, MS² fragmentation is rather scarce, consisting mainly in product ions deriving by loss of CO₂. Methylated phenolic acids present a richer fragmentation due to neutral losses of CH₃ prior to that of CO₂. On account of this, phenolic acid isomers, which differ for the position of hydroxyl groups on the aromatic ring, cannot be distinguished in HCD-based MS² experiments. However, hydroxycinnamic acid esters, due to the much higher ionization efficiency of acids compared to sugars, have been deeply studied for the distinction of isomers[32]. In particular, by comparing the relative abundances of specific fragments it is possible to distinguish which of the hydroxyl groups has been esterified. As stated by Fang et al. in their study on caffeoylquinic acid isomers[32], the relative ratios between Q₁, Q₂ and C₁ fragments can give information on which of the hydroxyl group of the quinic acid is esterified by caffeic acid (Figure 6).

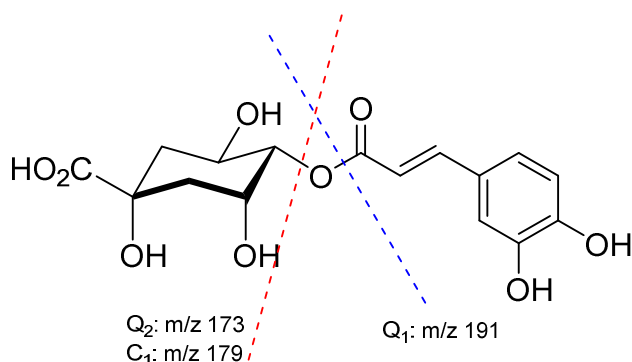


Figure 6. Fragmentation pathways of caffeoylquinic acid

Compound 136 and 137 have been tentatively identified as neochlorogenic acid (5-caffeoylquinic acid) since Q₁ and C₁ and its further fragmented ions have similar abundance, whereas compound 138 has been identified as chlorogenic acid (3-caffeoylquinic acid) by match of retention time and MS² spectrum of the available standard. Chlorogenic acid, differently from its isomer, shows a different ratio between the two fragments, with Q₁ (*m/z* 191.0563) being far more abundant than C₁ (*m/z* 179.0350). None of the three isomers presented high abundance of Q₂ ion (*m/z* 173.0455), which would have indicated the presence of cryptochlorogenic acid (4-caffeoylquinic acid). Based on those considerations, coumaroylquinic acid isomers were also discriminated. Compound 139 was tentatively identified as 5-coumaroylquinic acid, since it showed the highest relative abundance of C₁ fragment and its derivatives, while compounds 143-145 were identified as 3-coumaroylquinic acid, seeing that Q₁ was the base peak. Moreover, compound 140 was tentatively identified as 4-coumaroylquinic acid, since it presented Q₂ as the most abundant product ion. Complete data for identified phenolic acids in industrial hemp flowers are reported in Table S16.

4. Conclusions

The paper describes the development of an analytical platform for the identification of phenolic compounds based on HRMS data. The software Compound Discoverer 3.0 was implemented with a

comprehensive database of phenolic compound derivatives and the fragmentation patterns of base units for the semi-automatic identification of several classes of polyphenols. The method was then applied to industrial hemp extracts from the *Kompolti* variety, which is widely cultivated in Europe. In contrast to previous works on identification of industrial hemp antioxidant compounds, in which only few compounds were identified, in the present study a comprehensive profiling of flavonoid and phenolic acid derivatives was achieved, allowing the identification of 147 polyphenols belonging to the classes of flavonoids, proanthocyanidins and phenolic acids. The data processing method appears promising for the specific profiling of phenolic compounds in plant matrices as it allows the identification of a wide range of compounds which were never reported before. Nevertheless, efforts are still needed to include other polyphenol derivatives in the customized database with the purpose of obtaining information on a broader range of compounds. Finally, the chosen approach is applicable to other classes of structurally related compounds, such as lipids and peptides.

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Figure 1. Combination of flavonoid and phenolic acid derivatives generated for the polyphenol database

Figure 2: MS² spectra of quercetin *O*-pentoside *O*-hexoside obtained with methods *a-d* in positive and negative mode. Fragments in the *m/z* 400-600 range derive from the loss of one of the sugar, those in the *m/z* 200-300 range are generated by neutral losses of small molecules and ions that possess *m/z* below 200 derive from RDA ring openings and heterocyclic ring fissions.

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Figure 4. Comparison of the MS² spectra of standard biochanin A (*a*) and diosmetin (*b*) to compound *c*, tentatively identified as acacetin

Figure 5. B-type proanthocyanidins fragmentation pathways

Figure 6. Fragmentation pathways of caffeoylquinic acid

Highlights

- Compilation of a comprehensive database of 45,567 phenolic compound derivatives
- Comparison of 4 different mass-spectrometric methods for polyphenol fragmentation
- Development of an innovative data analysis workflow for phenolic compound identification
- Semi-automatic identification of 147 phenolic compounds in industrial hemp
- Development of a new Compound Discoverer approach for structurally related compounds

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

None