Authentication of true cinnamon (Cinnamomum verum) utilising direct analysis in real time (DART)-QToF-MS

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The use of cinnamon as a spice and flavouring agent is widespread throughout the world. Many different species of plants are commonly referred to as ‘cinnamon’. ‘True cinnamon’ refers to the dried inner bark of Cinnamomum verum J. S. Presl (syn. C. zeylanicum) (Lauraceae). Other ‘cinnamon’ species, C. cassia (Nees & T. Nees) J. Presl (syn. C. aromaticum Nees) (Chinese cassia), C. loureiroi Nees (Saigon cassia), and C. burmannii (Nees & T. Nees) Blume (Indonesian cassia), commonly known as cassia, are also marketed as cinnamon. Since there is a prevalence of these various types of ‘cinnamons’ on the market, there is a need to develop a rapid technique that can readily differentiate between true cinnamon (C. verum) and other commonly marketed species. In the present study, coumarin and other marker compounds indicative of ‘cinnamon’ were analysed using DART-QToF-MS in various samples of cinnamon. This method involved the use of [M + H]⁺ ions in positive mode in addition to principal component analysis (PCA) using Mass Profiler Professional software to visualise several samples for quality and to discriminate ‘true cinnamon’ from other Cinnamomum species using the accurate mass capabilities of QToF-MS.

Keywords: cinnamon; DART-QToF-MS; coumarin; PCA

Introduction

Cinnamon is a common spice that has been used around the world for many centuries and with lots of uses in cosmetics, food and pharmaceuticals. It is obtained from the dried inner bark of tropical evergreen trees of the genus Cinnamomum (Jayatilaka et al. 1995; Veliček 1999; WHO Monographs 1999; Tainter & Grenis 2001; Jana & Zdenka 2012; Wang et al. 2013). In the American and European markets, two varieties of cinnamon, Ceylon and cassia, are available. Ceylon cinnamon, also known as ‘true cinnamon’, is obtained from Cinnamomum verum J. S. Presl. (syn. Cinnamomum zeylanicum Nees) indigenous to Sri Lanka and southern India (Jayatilaka et al. 1995; Veliček 1999; WHO Monographs 1999; Tainter & Grenis 2001; Jana & Zdenka 2012; Wang et al. 2013). Cassia cinnamon has several origins, the important ones being Chinese cassia (Cinnamomum cassia Blume, syn. Cinnamomum aromati-cum Nees) cultivated mainly in southern China and Burma; Saigon cinnamon (Cinnamomum loureii Nees) from Vietnam; and Indonesian cassia (Cinnamomum burmannii Blume), mainly from Indonesia and the Philippines (Jayatilaka et al. 1995; Veliček 1999; WHO Monographs 1999; Tainter & Grenis 2001; Jana & Zdenka 2012; Wang et al. 2013). The chemical composition of volatile components varied significantly in these cinnamon samples. The quality of cinnamon depends on many factors such as botanical source, climatic conditions, and methods of harvesting and production. True cinnamon and cassia’s taste and smell vary due to the different chemical constituents. An important difference is in the amount of coumarin belonging to the benzopyrene family and occurs at higher concentrations in cassia cinnamon. Cassia cinnamon contains up to 1% coumarin, whereas true cinnamon contains only trace quantities at about 0.004% (Jayatilaka et al. 1995; Veliček 1999; WHO Monographs 1999; Tainter & Grenis 2001; Jana & Zdenka 2012). Coumarin is a hepatotoxic (Abraham et al. 2010) natural compound found in varied concentrations in different Cinnamomum species. Due to the high cost of true cinnamon, cassia cinnamon is often used as a substitute and it is therefore necessary to use analytical methods to discriminate between these two main types and to detect food adulteration.

These limitations necessitate an easy and fast method for the determination of coumarin levels and presence of other components especially in cinnamon-containing products. Conventionally, coumarin alone and/or in combination with other components were analysed using chromatographic and spectroscopic methods (He et al. 2005; Jana & Zdenka 2012; Li et al. 2013; Wang et al. 2013; Ballin & Sørensen 2014). These methods are often time-consuming.

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Recently, ambient MS has emerged as a promising analytical tool for the metabolomic fingerprinting of compounds having low molecular weights. In addition to food quality assessment or safety control, authentication is a challenging concern not only for food producers, regulatory bodies or scientific professionals but also for consumers. This novel methodology was applied for the identification of specific molecules from diverse samples such as metabolites, pharmaceuticals, food and chemicals. Direct analysis in real time (DART), a relatively new ionisation source for MS, ionises small-molecule components from different kinds of samples without any sample preparation and chromatographic separation (Chernetsova & Morlock 2011; Wang et al. 2014) and uses helium or nitrogen as carrier gases (Hajslova et al.; Wang et al. 2014).

The adaptation of DART-MS to analyse the raw materials of foods, botanicals and pharmaceuticals gives an unprecedented method for the rapid and efficient analysis of important compounds. Few studies namely, authentication of organic versus conventional crops (Novotná et al. 2012), beer origin recognition (Cajka et al. 2011), olive oil samples representing different quality grades from various countries (Vaclavik et al. 2009), animal fat recognition (lard and beef tallow) (Vaclavik et al. 2011), and botanical origin of cubeb fruits (Kim et al. 2011) have successfully combined DART-MS with multivariate analysis techniques to acquire metabolomic profiles related to foods and medicinal herbs. This novel methodology coupled with QToF-MS was used for qualitative identification and confirmation of chemical components from 35 authentic samples of four different species of cinnamon. Through PCA analysis of DART-MS data, marker compounds were identified that could help distinguish between 'true cinnamon' and other cinnamon samples. The method has an advantage of minimal sample preparation, simplicity in sample introduction, speed of analysis and high sample throughput under atmospheric pressure.

Materials and methods

Chemicals and plant samples

The standard compounds coumarin, cinnamyl alcohol, cinnamaldehyde, cinnamic acid and eugenol were purchased from Sigma (St. Louis, MO, USA) (Figure 1).

The samples analysed in this work were in the form of dried bark powders. In total, 35 samples comprised of nine samples of *C. verum* (NCNPR code numbers 3979, 3977, 3986, 3978, 3975, 3981, 3987, 3985 and 3997), 15 samples of *C. aromaticum* (NCNPR code numbers 9441, 9443, 9448, 9778, 9779, 9780, 9781, 9782, 9783, 9784, 9790, 9792, 9794, 9795 and 5227), five samples of *C. loureiroi* (NCNPR code numbers 9440, 9444, 9447, 3995 and 9446), and six samples of *C. burmannii* (NCNPR code numbers 9445, 9449, 9785, 9791, 9796 and 3996) were used for analysis (Wang et al. 2013). Authentic samples of *Cinnamomum verum* J. Presl (syn. *C. zeylanicum* Blume) (NCNPR code number 3997), *C. burmannii* (Nees & T. Nees) Blume (NCNPR code number 3995), and *C. loureiroi* Nees (NCNPR code number 3996) were obtained from Savory Spice Shop (Denver, CO, USA), and authenticated by macroscopic and microscopic methods by Dr Aruna Weerasooriya (Schneider 1921; Khatoon & Mehrotra 2009). An authentic sample of *C. cassia* (Nees & T. Nees) J. Presl (syn. *C. aromaticum* Nees) (NCNPR code number 5227) was purchased in China and authenticated by macroscopic and microscopic methods by Dr Vaishali C. Joshi (Schneider 1921). All these samples were deposited at the National Center for Natural Products Research (NCNPR), University of Mississippi, MS, USA.

Sample preparation

All standards (1 µg ml⁻¹) were prepared by dissolving in methanol. For this experiment all cinnamon samples were finely powdered then deposited on Dip-it™ tips (IonSense, Saugus, MA, USA). Excess powder was shaken off the Dip-it™ and the samples were then analysed sequentially using the 12 Dip-it™ holder and the built-in automated DART-SVP software. The samples were transferred to the optimised position in front of the DART gun exit. The sample was then desorbed from the surface of the glass rod for 10 s by helium, creating charged ions outside the instrument, during which time the spectral data were recorded. Each sample was run three times. During the sample preparation blanks were prepared consisting of all the steps mention above except for the addition of sample. After every three samples the blank sample was used. To perform a mass drift compensation for accurate mass measurements and elemental composition calculations, a drop of the reference solution was deposited on...
each Dip-it™ sample prior to the application of the cinnamon sample.

Instrumentation and experimental conditions

DART-QToF/MS

DART-QToF/MS system consisting of a DART ion source (DART SVP software version 4.3.3b, IonSense, Saugus, MA, USA) installed on a QToF/MS, high-resolution time-of-flight MS. The DART source replaces the standard ESI source supplied with the QToF-MS. The operating conditions of the DART ion source were as follows: positive-ion mode; helium flow: 2.5 l min⁻¹; grid voltage: 350 V and gas heater set to 500°C. The MS analysis was performed with a QToF-MS (Model Number G6530A, Agilent Technologies, Santa Clara, CA, USA) equipped with an atmospheric pressure ionisation (API) interface using the following parameters: drying gas (N₂) flow rate, 3.0 l min⁻¹; drying gas temperature, 300°C; capillary, 1000 V; fragmentor voltage, 125 V. All the operations, acquisition and analysis of data were controlled by Agilent MassHunter Acquisition Software Ver. A.06.00 and processed with MassHunter Qualitative Analysis software Ver. B.06.00. Each sample was analysed in positive mode in the range of m/z = 50–1000. Accurate mass measurements were obtained by means of reference ion correction using reference masses at m/z 121.0509 (protonated purine) and 922.0098 [protonated hexakis-(1H,1H,3H-tetrafluoropropoxy) phosphazine or HP-921]. The compounds were confirmed in each spectrum (Figure 1). For this purpose, the reference solution of the two compounds was prepared and a drop was deposited on each Dip-it™ sample. The distance between the DART gun exit and MS inlet was 10 mm. Sample introductions were carried out automatically using Dip-it™ samplers (IonSense). To perform a mass drift compensation for accurate mass measurements and elemental composition calculations, a reference mass solution was introduced manually to each sample.

Data processing and chemometric analysis

MassHunter Workstation software, including Qualitative Analysis (version B.06.00), was used for processing raw data, including molecular feature extraction, background subtraction, data filtering and molecular formula estimation (using accurate mass of all isotopes, their relative abundances and all detected adducts with their measured isotopes). To perform subtraction of molecular features (MFs) originating from the background, analysis of a blank sample (methanol) was carried out under identical instrument settings and background MFs were removed. The data were obtained by averaging the intensities of the mass spectra recorded during the exposure of the sample to the DART gas beam background ions were subtracted and the mass drift was corrected. Potential markers were selected after careful examination of DART-MS spectra; the ions with significant intensities (threshold 1% of the ion with the highest intensity in respective mass spectrum) were chosen. All the selected ions are shown in Table 1. The results of repeated measurements were averaged. In the follow-up step, chemometric tools were employed for the processing of MS data. The MassHunter Qualitative export tool was then extracted compound list for each file was exported as Compound Exchange Format (.cef) file and imported into Mass Profiler Professional (MPP Version 12.6.1) statistical software, which aligned, normalised, visualised and filtered the molecular features (MFs). The resulting feature files for each sample were processed by analysis of variance (ANOVA) and PCA

<table>
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<th>Number</th>
<th>Formula</th>
<th>Experimental m/z value</th>
<th>Calculated m/z value</th>
<th>Error (mDa)</th>
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<td>147.0441</td>
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<td>237.1849</td>
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</tr>
<tr>
<td>13</td>
<td>C₁₁H₂₀₂</td>
<td>235.1679</td>
<td>235.1693</td>
<td>1.4</td>
<td>–</td>
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</table>
Results and discussion

DART-QToF-MS analysis of cinnamon

A recently developed ionisation source for MS, termed direct analysis in real time (DART), was coupled to a ToF MS, making it possible to identify rapidly and accurately analyse the chemical components in botanicals at atmospheric pressure, with minimal sample preparation or processing. The DART utilises electronically excited helium atoms as an ionising plasma source. These excited atoms can ionise samples directly under atmospheric conditions for subsequent MS analysis.

The DART-MS instrumental parameters, such as gas heater temperature, helium pressure, linear rail speed, distance of DART source from the MS orifice and the grid voltage were varied to determine the optimal ionisation conditions for standards. Reproducibility experiments were performed in replicates and the method was checked by determining precision on a same instrument. The low % RSD (< 1) values indicate that the method is reproducible.

Applications of DART in analysis of cinnamon samples

An important advantage of DART is that materials can often be analysed directly without solvent extraction. This allows for the possibility to screen samples rapidly with minimal preparation steps. Careful analysis is important since the lack of a separation step makes the DART-MS approach more vulnerable to false findings such as detecting major components that are in the surrounding environment but not in the sample. DART produces simple mass spectra characterised by \([M + H]^+\) (protonated molecule) in positive-ion mode. Both positive- and negative-ionisation modes were used for the analysis of cinnamon compounds. Positive-ionisation mode enabled better differentiation between cinnamon samples than the results from the negative mode.

The mass spectra of C. verum, C. cassia, C. loureiroi and C. burmannii obtained in the positive mode are shown in Figure 2, which displays the baseline-corrected data. Two subgroups corresponding to mass-to-charge (m/z) ranges 130–170 and 195–240 were characterised as phentylpropane and sesquiterpeneprope compounds, respectively. In the phenylpropane group, peaks at m/z 133.0644 and 147.0436 were identified as cinnamaldehyde and coumarin, respectively, according to high-resolution accurate mass measurement and comparison with reference standards; peaks at m/z 163.0749 and 164.0701 were tentatively characterised as methyl cinnamate and aminocinnamic acid, respectively, on the basis of their accurate mass, isotope abundances and spacing. Additional signals at m/z 135.08 \([M + H]^+\), 149.05 \([M + H]^+\), 165.09 \([M + H]^+\), and 177.09 \([M + H]^+\) were determined to be the detected ions of cinnamyl alcohol, cinnamic acid, eugenol and cinnamyl acetate, respectively. For the sesquiterpene group, four compounds were tentatively characterised as caryophyllene/copaene/guaiene (C\(_{15}\)H\(_{24}\)), dehydro-sesquiterpenes (C\(_{15}\)H\(_{22}\)), dehydro-sesquiterpene oxide (C\(_{15}\)H\(_{23}\)O), sesquiterpene oxide (C\(_{15}\)H\(_{24}\)O), and caryophyllene epoxide, respectively, corresponding to the peaks at m/z 203.1785, 205.1944, 217.1589, 219.1739 and 221.1886 (Table 1). Two peaks at m/z 235.1679 and 237.1836 related to chemical formula as C\(_{15}\)H\(_{22}\)O\(_2\) and C\(_{15}\)H\(_{24}\)O\(_2\), respectively, were tentatively determined as further oxidised sesquiterpenes. Cinnamomum loureiriti had a high percentage of trans-cinnamaldehyde. C. aromatycum contained abundant quantities of methyl cinnamate and guaiene (C\(_{15}\)H\(_{24}\)) compared with C. loureiriti, C. burmannii and C. verum. C. burmannii contained significant amounts of coumarin in comparison with the other three types of cinnamon (Table 2). In all these four species of cinnamon the variation of components is different, which may be caused by differences in habitat, harvest season and extraction techniques (Li et al. 2013). It has been reported that the bark of C. burmannii contains higher amount of essential oils, primarily 1,8-cineole, α-terpineol, camphor, terpinen-4-ol, borneol, α-pinene, β-caryophyllene (C\(_{15}\)H\(_{24}\)), and p-cymene (Al-Dhubiab 2012).

Principal component analysis (PCA)

PCA is a frequently employed unsupervised multivariate analysis technique enabling data dimensionality reduction, while retaining the discriminating power in the data. It is used as a quality control tool to provide an idea of how the data cluster and identifies sample outliers. PCAs of the entities that varied in amount were used and confirmed the distinctive grouping of the data (Figure 3). The amount of fold-change (increase) in the concentration of any given compound was determined first. This analysis identified entities with large abundance differences between the selected data classes, that is, those that differed in concentration by two-, three-, four-fold, and so forth between pass and fail cinnamon samples. Next, ANOVA was used to determine if the differences between those compounds that met the fold-change criteria that were statistically significant. Using a probability \(p < 0.05\), the 157 entities from the frequency filter were reduced to seven significant compounds. The seven compounds with the lowest \(p\)-values and highest fold-changes (most significant with greatest abundance differences) were selected.
The separation was observed in the first two principal components (PC-1 and PC-2 where 76% of the variance was explained). Examination of the scores and loading plots for PC-1 versus PC-2 showed that compounds cinnamaldehyde, coumarin, methyl cinnamate, aminocinnamic acid and three sesquiterpenes were responsible for the separation. The circled molecular formulas in Figure 2 were used as markers for the discrimination of cinnamon.

Table 2. Typical relative abundance (RA, n = 3) and relative standard deviations (RSDs) of seven marker compounds observed in DART mass spectra of different Cinnamon samples.

<table>
<thead>
<tr>
<th>Number</th>
<th>m/z</th>
<th>Molecular formula</th>
<th>Number 3996, C. burmannii</th>
<th>Number 3997, C. verum</th>
<th>Number 5227, C. aromaticum</th>
<th>Number 3995, C. loureiroi</th>
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</thead>
<tbody>
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<td>1</td>
<td>133.06</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;O</td>
<td>14.5 ± 0.26</td>
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<td>29.8 ± 0.13</td>
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</tr>
<tr>
<td>2</td>
<td>147.04</td>
<td>C&lt;sub&gt;7&lt;/sub&gt;H&lt;sub&gt;9&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>100 ± 0.11</td>
<td>20.2 ± 0.43</td>
<td>85.2 ± 0.07</td>
<td>57.9 ± 0.2</td>
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<tr>
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<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>69.4 ± 0.08</td>
<td>100 ± 0.14</td>
<td>87.5 ± 0.11</td>
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<tr>
<td>4</td>
<td>164.07</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;9&lt;/sub&gt;NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>51.5 ± 0.12</td>
<td>16.3 ± 0.16</td>
<td>52.3 ± 0.08</td>
<td>20.4 ± 0.42</td>
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<tr>
<td>5</td>
<td>205.19</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;</td>
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<td>9.1 ± 0.29</td>
<td>7.6 ± 0.5</td>
<td>38 ± 0.14</td>
<td>6.5 ± 0.26</td>
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</table>

Figure 2. Comparative mass spectra of C. burmannii, C. verum, C. cassia and C. loureiroi using DART-MS spectral data in positive-ion mode. The circled compounds were used for PCA analysis.
samples. The separation is due to mainly PC-1. PC-2 did not have much effect on the separation of compounds because the same samples show a less reproducible value when compared with PC-1 value. All the marker compounds are present in all the authentic samples of each species. Their differentiation is determined by the relative concentration of the markers in each species. The samples of *C. verum* (numbers 3997, 3979, 3977, 3986, 3978, 3975, 3981, 3987 and 3985) were distinguished from other cinnamon samples. Sample numbers 3985, 3986, 3987, 3997 and 3981 were closely related, and the other sample numbers 3975, 3978, 3977 and 3979 were positioned apart due to specific patterns or composition of compounds. The differences between these two groups are mainly based on the concentration of sesquiterpenes. Table 2 shows those differences, notably with coumarin (m/z 147.04), is significantly lower in *C. verum* than all other species. In addition, methyl cinnamate (m/z 163.07) and cinnamaldehyde (m/z 133.06) were abundant in *C. verum* than in *C. burmannii* and *C. loureiroi*. In all *C. verum* samples analysed, the three sesquiterpenes and coumarin content were very low compared with methyl cinnamate and cinnamaldehyde content. The *C. aromatricum* samples contained high amounts of three sesquiterpenes, coumarin and methyl cinnamate and low amounts of cinnamaldehyde. The samples of *C. burmannii* contained high amounts of coumarin, methyl cinnamate and amino cinnamic acid but low amounts of cinnamaldehyde.

**Figure 3.** Three-dimensional (3D) PCA and PCA score plots of *C. verum*, *C. cassia*, *C. loureiroi* and *C. burmannii* using DART-MS spectral data in positive-ion mode.
and sesquiterpenes. The C. loureiroi samples contained high amounts of cinnamaldehyde, coumarin and methyl cinnamate. Based on these differences in the content of seven markers the separation of C. verum samples away from other species of cinnamon was observed.

Using the positive ESI accurate mass data, a molecular formula for each marker compound was generated. The accurate mass information available helped to confirm tentative identifications for seven compounds. Using the positive ESI accurate mass data, a molecular formula for each marker compound was generated. The accurate mass information available helped to confirm tentative identifications for seven compounds, with a mass accuracy better than 2 ppm. Knowing the identity of these compounds, routine analysis of cinnamon could be done by DART with a single quadruple MS or other direct probe MS techniques. This would be much less expensive while maintaining the speed of the DART methodology employed here. This approach will be very useful to predict whether ‘true cinnamon’ has been adulterated with other cinnamons.

Conclusions
DART-QToF-MS was used for direct rapid analysis and discrimination of cinnamon samples, without any sample preparation. In positive-ion mode, the [M + H]+ ion was easily identified based on the accurate mass measurement. Through the use of this newly developed DART-QToF-MS method it was possible to analyse rapidly multiple authenticated and commercial samples of cinnamon. The ability to employ high-resolution MS provided the capability to identify tentatively many of the major constituents within the various cinnamon samples. MS of these constituents in comparison with authentic standards would provide a confirmatory identification. Coupling the resultant data with further PCA analysis provided a model which clearly discriminates populations of ‘true cinnamon’ C. verum from other commonly marketed cinnamon species. This can be an important aid to a commodity used worldwide. These results highlight the benefits of employing DART-QToF-MS for the analysis of botanicals. A simple chemical profiling and PCA analysis for cinnamon samples using DART-MS might be useful in quality control of ‘true cinnamon’.

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