Detection of capsinoids by ESI-mass analysis

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Abstract

The substances responsible for the pungency of *Capsicum* are a group of alkaloids known as capsaicinoid. Recent studies have also revealed the presence of a novel group of non-pungent capsaicinoid-like substances named capsinoids. These compounds have structures similar to capsaicinoids and produce the same biological effects but without the undesirable irritation caused by the pungency, turning into molecules with potential applications in areas such as medicine. The capsinoid biosynthesis pathway is being investigated to trying to clarify the genetic and metabolic mechanisms of biosynthesis. The development of a more sensitive and selective analytical method to identify these compounds may be useful to select pepper cultivars that produce these compounds and help gain increase knowledge about their biosynthesis and biological activity.

Keywords: *Capsicum*, capsinoid, pepper, mass spectrometry

Introduction

Capsaicinoids are compounds responsible for pungency in pepper (*Capsicum* spp) that are synthesized by condensation of a common vanillyl moiety and a variable fatty acid (Nelson et al. 1923). These secondary metabolites exhibit pharmacological effects such as analgesia, anticancer, antioxidant and antiobesity activities among other (Reyes-Escogido et al. 2011). However, their use as ingredients in certain foods and pharmaceuticals has been limited by pungency, the main feature that makes them popular as a spice. Recently, another capsaicinoid-like substances, named capsinoids, have been discovered in pepper fruit extracts from different cultivars, such as 'CH-19 Sweet'(*C. annuum*) (Yazawa et al. 1989), 'Zavory Hot', 'Aji Dulce', 'Belice Sweet' (all of them *C. chinense*) (Tanaka et al. 2010), and 'SR211'(*C. annuum*) (Koeun et al. 2013). So far, capsiate (4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate), dihydrocapsiate (4-hydroxy-3-methoxybenzyl 8-methylnonanoate) and nordihydrocapsiate (4-hydroxy-3-methoxybenzyl 7-methyloctanoate) (Kobata et al. 1998; Kobata et al. 1999) have been isolated in pepper fruit extracts. Fundamental chemical structure and the biologic activity of these compounds are very similar to the capsaicinoids. Unlike capsaicinoids, capsinoids are synthesized by the condensation of a variable fatty acid and a vanillyl alcohol moiety, in addition, are non-pungent, producing the same biological effects but without the undesirable irritation caused by the pungency, turning into molecules with potential applications in areas such as medicine (Sasahara et al. 2010). Many analytical methods have been developed to determine capsaicinoids using gas (GC) and high-performance liquid chromatography (HPLC) coupled to UV-Visible (UV) or mass spectrometry (MS) detection (Schweiggert et al. 2006; Garcés-Claver et al. 2006). However, a limit number of methods has been developed to determine the capsinoid compounds, using most of them UV detection. Significant structural information can be obtained from electrospray ionization (ESI)-(MS), quadrupole time-of-flight (QTOF) and ESI-ion trap-MS that provide molecular fragmentation patterns. These techniques can be used to identify unknown capsinoid-type molecules and also to confirm the identification of the three known capsinoids in vegetable matrices, which could be difficult due to the low concentrations present and the complexity of the matrix.

Therefore, the aim of this study has been the optimization of a mass detection method by high-resolution tandem mass spectrometry (QTOF and ion trap MS analyzer) that allows accurate *m/z* measurements of capsinoid ions and their product ions and the characterization of the fragmentation patterns of capsiate and dihydrocapsiate.

Material and Methods

Chemical and Reagents

Vanillin, anhydrous pyridine, *t*-butyl-dimethyl silyl chloride and di-isobutyl aluminum hydride (1M in toluene) were purchased from Sigma-Adrich (St. Louis, MO). *Trans*-8-methyl-6-nonenoyl chloride was purchased from Ambinter (Greenpharma S.A.S., Orleans, France) and 8-methyl-nonanoic acid was obtained from Acros Organics (New Jersey, USA). Ethyl acetate (≥ 99.8%, HPLC-grade) and n-hexane (≥ 95%, HPLC-grade) were obtained from VWR International L.L.C. (Radnor, Pensilvania, USA). Magnesium sulfate anhydrous (96%), Copper (II) sulfate pentahydrate (99.0-100.5%), hydrochloric acid (37%), and ethanol (99.5%) were purchased from Panreac Química (Barcelona, Spain).

Synthesis of capsiate and dihydrocapsiate standards

The synthesis of capsiate and dihydrocapsiate were accomplished at the University of Cádiz (UCA) (Cádiz, Spain). Capsiate was synthesized from the esterification of reduced and protected vanillin with the corresponding acyl chloride. Hydroxyl group of vanillin was protected with *t*-butyl-dimethyl silyl chloride, followed by a reduction of the aldehyde group with di-isobutyl aluminum hydride (1M in toluene). The silylated reduced vanillin was esterificated with the corresponding acyl chloride to obtain the silylated capsiate. Capsiate was desilyated with 0.25M HCl/ethanol mixture (1:5). Similar procedure was employed for the synthesis of dihydrocapiate standard. Proton and carbon nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectroscopy (UCA) was used for the structural elucidation of the synthesized capsiate and dihydrocapsiate.

ESI-MS^2(QTOF) and ESI- MS^n(Ion trap) analysis

Capsiate and dihydrocapsiate standard solutions (10 μM, 70% methanol and 0.1% formic acid) were detected by direct injection with a syringe pump (Cole-Parmer Instrument Co., Vernon Hills, IL, USA) operating at 4 μLmin^{-1}, in a QTOF and ion trap mass spectrometer equipped with an ESI source (MicroTof-Q and Esquire 3000 plus, Bruker Daltonics, Bremen, Germany). ESI-MS^2(QTOF) analysis was carried out in positive ion mode, with capillary and endplate offset voltages of 4800 and -500 V, respectively, and using N_2 as collision gas. Collision cell energy was set to 10 eV, with an isolation width for the precursor ion of 4 *m/z* units. The nebulizer (N_2) gas pressure, the drying (N_2) gas flow rate and the drying gas temperature were 0.7 bar, 4.0 Lmin^{-1} and 200ºC, respectively. The mass axis was calibrated using Na-Formate adducts [10 mM NaOH, 2.5% (v/v) formic acid and 50% (v/v) 2-propanol] that were introduced by direct injection.

ESI-MS^n(Ion trap) analysis was carried out in positive mode, with capillary and endplate offset voltages of 4000 and -500 V, respectively and using He as the collision gas. Spectra were acquired in the *m/z* 50–500 range at the Standard/Normal scan mode. For MS^2 spectra the $[M+Na]^+$ ions were chosen as precursors, while product ions with 159 and 137 *m/z* (characteristic of capsinoid fragmentation) were used as precursors for MS^3 analysis with an isolation width of 4 *m/z* and an amplitude voltage of 0.45 V. Bruker Daltonik software packages micrOTOF Control v.2.3, Esquire Control v.5.3 and Data Analysis v.4.0 were used to control the MS apparatus and process data, respectively.

Results and Discussion

The aim of the study was to optimize the mass detection of capsinoid compounds, for this purpose capsiate and dihydrocapsiate standards were synthesized. The procedure used for the synthesis was previously developed by Barbero et al. (2010). The structures of the capsiate and dihydrocapsiate synthesized were confirmed by NMR analysis. NMR data for the two standards matched the results obtained by Barbero et al. (2010).

ESI-MS^2 spectra of capsiate and dihydrocapsiate were obtained by direct injection of standard solutions (10µM) on the QTOF and the ion trap mass analyzers. For both mass analyzers, the major peaks observed in the ESI-MS spectra were the $[M + Na]^+$ molecular ions at the *m/z* 329 and 331, corresponding to sodiated capsiate and dihydrocapsite molecules, respectively. These ions were selected as precursor for the ESI-MS^2 experiments. Figure 1 shows the ESI-MS^2 spectra obtained for capsiate and dihydrocapsiate. For both capsinoids, the most intense product ion was observed at *m/z* 159.0 corresponding to the sodiated vanillyl ring shared by all capsinoids. Also, other minor product ions detected corresponding to the vanillyl ring were *m/z* 137.1 and 177.1, generated by the protonated vanillyl molecule and the different fragmentation of the sodiated vanillyl ring, respectively. Product ions at *m/z* 193.1 and 195.1, for capsiate and dihydrocapsiate, respectively, indicated the acyl chain residues. This is the first time that the fragmentation patterns of capsiate and dihydrocapsiate has been carried out.

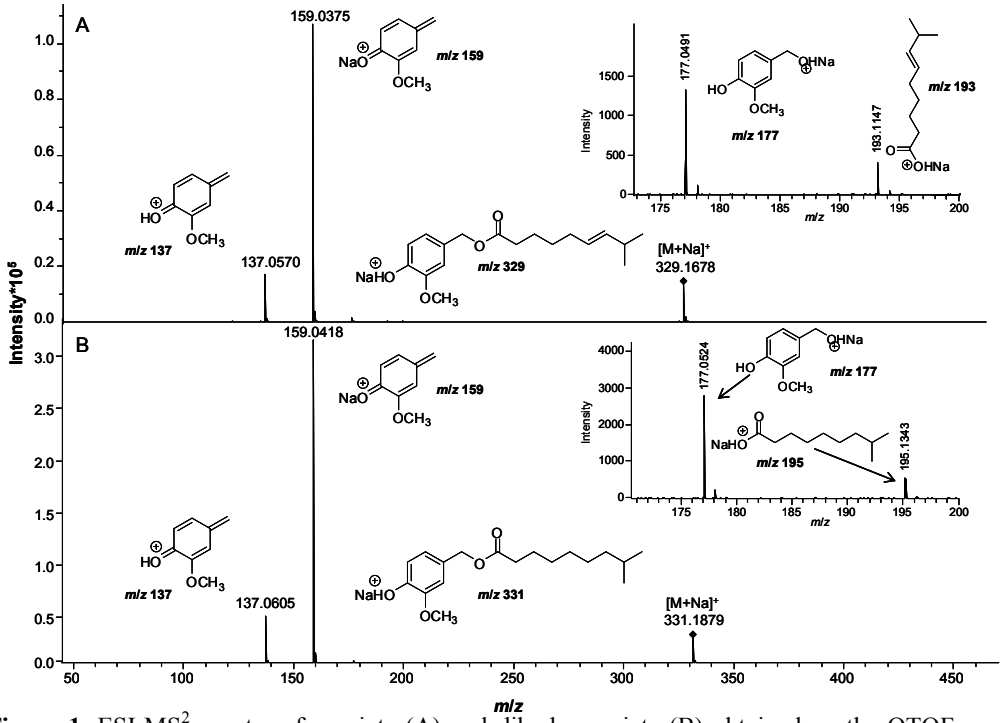

Figure 1. ESI-MS^2 spectra of capsiate (A) and dihydrocapsiate (B) obtained on the QTOF mass spectrometer in positive ion mode. Proposed fragmentation patterns for capsiate (A) and dihydrocapsiate (B).

The product ions with a 137 *m/z* obtained by the ESI-MS^2 analysis of capsiate and dihydrocapsiate standards were used as the precursors for MS^3(Ion trap) analysis. The major peaks observed in the MS^3 spectra were the molecular ions at the *m/z* 122.0, 79.2, and 107.0. Similar fragmentation patterns were observed for further fragmentation of the protonated ion at *m/z* 137

obtained of MS^2 experiment for capsaicin (Schweiggert et al. 2006), that corresponding to the common vanillyl ring.

The large number of capsaicinoids found in pepper fruit extracts (Schweiggert et al. 2006) can provide indications of possible occurrence of similar number of capsinoids in pepper fruit extracts. This study opens the possibility of applying ESI-MS(QTOF) analyses to identify unknown capsinoids in pepper fruit extracts. The fragmentation patterns obtained supply valuable information for further characterization of unknown capsinoid-type compounds.

Acknowledgements

This study was supported by the Spanish Ministry of Science INIA-FEDER (RTA2011-00118-C02-01) and by the Aragon Government (Group A16).

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