

Chapter 2

Electrospray Ionization Traveling Wave Ion Mobility Spectrometry Mass Spectrometry for the Analysis of Plant Phenolics: An Approach for Separation of Regioisomers

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Abstract The use of ion-mobility spectrometry (IMS) coupled to mass spectrometry (IMS–MS) for biomolecule analyses has steadily increased over the past two decades, and is now applied to both proteomic and metabolomic investigations. This chapter describes the application of traveling-wave ion-mobility spectrometry–mass spectrometry (TWIMS–MS) to the analysis of a selection of bioactive phytochemicals used in dietary supplements. Applications include the analysis of grape seed proanthocyanidins and the structural characterization of bioactive constituents of dietary supplements using TWIMS-MS in conjunction with tandem mass spectrometry. We also discussed is the application of TWIMS-MS for the gas-phase mobility separation of structural isomers and the estimation of collision cross sections for a small selection of phenolic compounds from hop. Recent applications of IMS–MS to a broad range of biomolecule measurements have demonstrated that IMS–MS has emerged as a powerful analytical technique capable of providing the separation space necessary to analyze highly complex samples. We give a perspective on emerging applications of IMS–MS for small molecule and biopolymer applications. The combination of devices that allow real-time monitoring of living systems using IMS–MS is an exciting avenue of facilitating system-biology experiments. The future of IMS–MS is bright and full of opportunities.

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2.1 Introduction

There is increasing evidence that plant phenolics have health benefits which may, at least partially, stem from their antioxidant and radical scavenging activity [1–3]. Considering the increasing interest in plant phenolics as nutraceuticals, comprehensive profiling methods for plant extracts are highly needed. We report on the characterization of plant phenolics using traveling-wave ion-mobility spectrometry–mass spectrometry (TWIMS–MS) and emphasize the structural analysis of plant secondary metabolites that are commonly found in over-the-counter dietary supplements.

The use of ion-mobility spectrometry (IMS) coupled to mass spectrometry (IMS–MS) for biomolecule analyses has steadily increased since the 1990s. Many applications describe IMS–MS for studying peptides and proteins and their folding behaviors in the gas phase [4–10]. IMS–MS has also been used for assessing synthetic polymers [11, 12]. More recently, IMS–MS has been described as a powerful addition to the arsenal of tools for the structural analysis of small molecules including drugs, metabolites, lipids, carbohydrates, phytochemicals, and other natural products [13–19]. Comprehensive reviews are available that describe in detail the principles and applications of IMS–MS [20–22]. Briefly, in IMS ions are separated according to their charge state, shape, and size. IMS systems function as gas-phase separation devices. IMS uses nondestructive low-energy collisions to separate ions predominately on the basis of ion-neutral collision cross sections. Ion-mobility separations in the gas phase have considerably lower resolution compared to the resolution that can be achieved with modern condensed phase chromatographic separation technologies. However, the separation of ions occurs several orders of magnitudes faster than separations based on liquid chromatographic techniques; ion-mobility separations usually occur on a time scale of milliseconds compared to the seconds to hours in chromatographic separations. MS measurements occur in the microsecond range and, as such, are nested within the IMS experiments [23]. IMS–MS experiments allow real-time separations of the components of complex mixtures and provide access to three-dimensional (3D) analytical information, namely shape, mass, and abundance. The combination of IMS with MS results in two-dimensional plots of drift time (t_d) versus m/z . The three-dimensionality of TWIMS–MS datasets is best captured in so-called driftscope images that contain information regarding the drift time (t_d , in ms) and m/z values displayed in a nested fashion, t_d as function of (m/z), with ion abundances given in a color-coded style. These images enable the extraction of distinct features that otherwise would get lost or overlap in crowded spaces of traditional mass spectra. A unique feature of IMS–MS is the ability to conduct drift time measurements that allow the calculation of collision cross sections (CCSs) for low- and high-molecular-weight molecules and the possible separation of isomeric analytes, which is not possible solely with MS-based techniques [13–15].

IMS–MS systems are available in many different instrument configurations. In the traditional configuration, the drift tube-based IMS device is placed between the ionization source and the mass analyzer [20]. The recent advent of a commercial

IMS–MS system, in which a traveling-wave IMS device has been integrated into a hybrid quadrupole-orthogonal acceleration time-of-flight (TOF) mass spectrometer, opens new possibilities for the structural characterizations of small molecules and biopolymers [24]. In this contribution, we describe the application of TWIMS–MS for the analysis of a selection of bioactive phytochemicals used in dietary supplements. We first give a brief description of the TWIMS–MS instrument used. Then, we discuss several applications of TWIMS–MS: (1) the analysis of a biopolymer mixture, namely grape seed proanthocyanidins; (2) the structural characterization of bioactive constituents of dietary supplements using TWIMS in conjunction with tandem mass spectrometry; and (3) the gas-phase mobility separation of structural isomers and the estimation of collision cross sections for a small selection of phenolic compounds from hop. We conclude with a brief review of recent developments and applications, and provide a perspective on the emerging application of IMS–MS for small molecule and biopolymer applications.

2.2 Ion-Mobility Mass Spectrometry Using an Electrospray Ionization Quadrupole Traveling-Wave Ion-Mobility TOF Instrument

Many different instrument designs have been described that combine IMS with different types of MS analyzers [20]. The availability of a commercial hybrid system that integrates traveling-wave ion-mobility separation with a quadrupole TOF analyzer makes the technology accessible to a wider research community. For the applications described here, we used a Waters Synapt G2 HDMS instrument equipped with an electrospray ionization (ESI) source. This instrument has the following configuration: a quadrupole mass filter, the TriWave™ section consisting of three traveling-wave (T-wave) devices (Trap T-wave, Ion mobility separation (IMS) T-Wave, and Transfer T-wave) and an orthogonal acceleration (oa) TOF analyzer (Fig. 2.1). The traveling-wave IMS device is a radio-frequency (RF) ion guide based on a stack of ring electrodes. The RF voltage confines the ions radially. By applying a continuous series of DC voltage pulses traveling along the stacked ring electrodes, ions are moved through the gas-filled guide. The ability of an ion to travel along with the DC pulse through the gas-filled T-wave ion guide will depend on the ion's size, charge, shape, and the interaction cross section between the ion and background gas. Instrumental parameters that affect the ability of an ion to move with the traveling wave are the amplitude of the wave, the travel velocity of the wave, and the type and pressure of the background gas. Applying an optimized set of parameters, high-mobility ions will travel with the wave and pass through the ion guide faster than ions with low mobility, which roll over the top of the wave and as a result spend more time in the device [24].

The TriWave™ section of this instrument consists of three traveling-wave-enabled stacked ring ion guides. This configuration allows for unique fragmentation experiments (Table 2.1). The trap ion guide is used for the accumulation of ions and

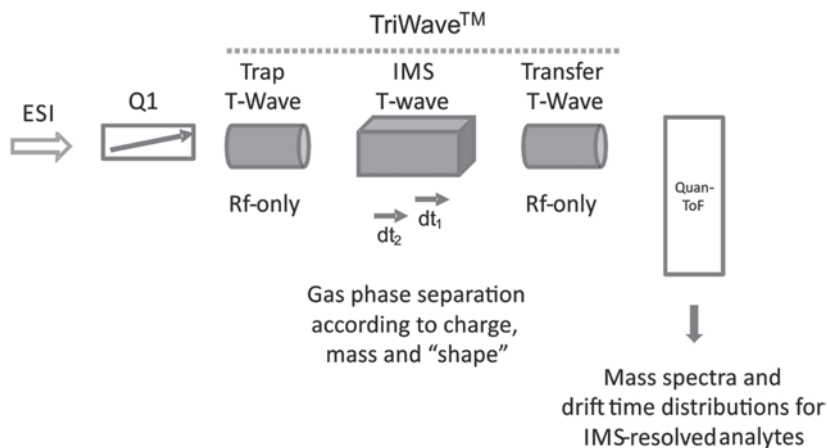


Fig. 2.1 Conceptual diagram of the commercially available TWIMS–MS instrument (Waters Synapt G2 HDMS instrument) operated in the mobility time-of-flight (TOF) mode. This operating mode is used for gas-phase mobility separation of ions in combination with high-resolution mass spectrometry. This mode also enables the extraction of drift times for the estimation of collision cross sections after calibrating the traveling-wave ion-mobility separator. In mobility-TOF mode, the quadrupole (Q1) analyzer is operated in the transmitting mode, the trap and transfer devices serve at radio-frequency (Rf)-only ion guides. *T-wave*, traveling wave; *IMS*, ion mobility spectrometry; *dt*, drift time

release of ions as packets into the ion-mobility separation device. The transfer ion guide conveys the mobility-separated ions to the orthogonal acceleration (oa) TOF analyzer. Fragmentation can take place either in the trap, in the transfer device, or in both devices. A detailed description of the working principle and the design of the traveling-wave ion-mobility separator have been published previously by Giles et al. [24, 25]. Details on the theoretical background on classical IMS–MS and the adaption to traveling TWIMS–MS have also been described in several recent papers [24–27]. In the following sections, we describe the use of TWIMS–MS experiments for the structural characterization of plant phenolics.

2.3 TWIMS–MS Analysis of Biopolymers: Application to Grape Seed Proanthocyanidins

Grape seed extracts have been extensively studied by diverse mass spectrometric techniques. In most cases, the mass spectrometric analyses were accompanied by laborious and extensive chromatography of the highly complex grape seed proanthocyanidin mixtures [28–30]. Considering the current interest in grape seed proanthocyanidins as nutraceuticals in biomedical applications, comprehensive and fast profiling of grape seed extracts is highly desirable. The analysis of proanthocyanidins by ESI–MS is challenging due to the overlapping of ion signals of constituents

Table 2.1 Operating modes of the TWIMS–MS instrument utilizing the TriWave™ section

Mode	TriWave™ usage			Products
	Trap	TWIMS device	Transfer	
Mobility-ToF	Ion guide only	Ion-mobility separation	Ion guide only	Ions are separated according to their mobility; drift time measurements enable collision cross-section estimations after calibration of the TWIMS device
Trap fragmentation	Elevated energy	First-generation fragment ions	Ion guide only	First-generation fragment ions are ion mobility separated
Transfer fragmentation	Storage device only	Precursor ions	Elevated energy	Precursor ions are separated according to their mobility. First-generation fragment ions align with precursor ion drift time
Time-aligned parallel (TAP) fragmentation	Elevated energy	First-generation fragment ions	Elevated energy	First-generation fragment ions are separated according to their mobilities. Activation in the transfer device results in second-generation product ions which are time-aligned to the respective first-generation product ion precursor

of the highly complex mixtures of proanthocyanidins oligo- and polymers differing in length (i.e., degree of polymerization or DP), subunit composition, and type of linkage between flavanol units [29–33].

The three-dimensionality of TWIMS–MS dataset is highlighted in Fig. 2.2a–d. ESI–TWIMS–MS driftscope images (drift time versus m/z ; color coding: white most to blue least abundant ions) of a typical grape seed proanthocyanidin preparation are shown (Figs. 2.2a, c). What makes these images remarkable is (i) the separation of the ions into distinct charge state groups which are denoted as (+1) and (+2) and (ii) the separation of the proanthocyanidin oligomers into distinct ion clusters. The sodium adducts of the proanthocyanidin oligomer ions displayed shorter drift times than the corresponding protonated molecular ions (Fig. 2.2a).

In the TWIMS–MS driftscope image depicted in Fig. 2.2a, the gas-phase mobility separation of singly protonated proanthocyanidin ions from the doubly protonated proanthocyanidin ions is highlighted. The two charge state groups are denoted with (+1) and (+2). In Fig. 2.2b, a section of the ESI mass spectrum (m/z 500–1,400) is depicted. Singly charged ion signals dominate the mass spectrum. Due to the mobility separation of singly and doubly charged ions, it is possible to extract distinct ion clusters, which helps with the analysis of overlapping ion signals. For instance, extraction of the singly protonated ions of the procyanidin trimer (PC3, $[M+H]^+$, m/z 867.2) and the doubly protonated ions of the procyanidin hexamer (PC6, $[M+2H]^{2+}$, m/z 866.2) results in mass spectra that show baseline resolved isotope clusters (Fig. 2.2c).

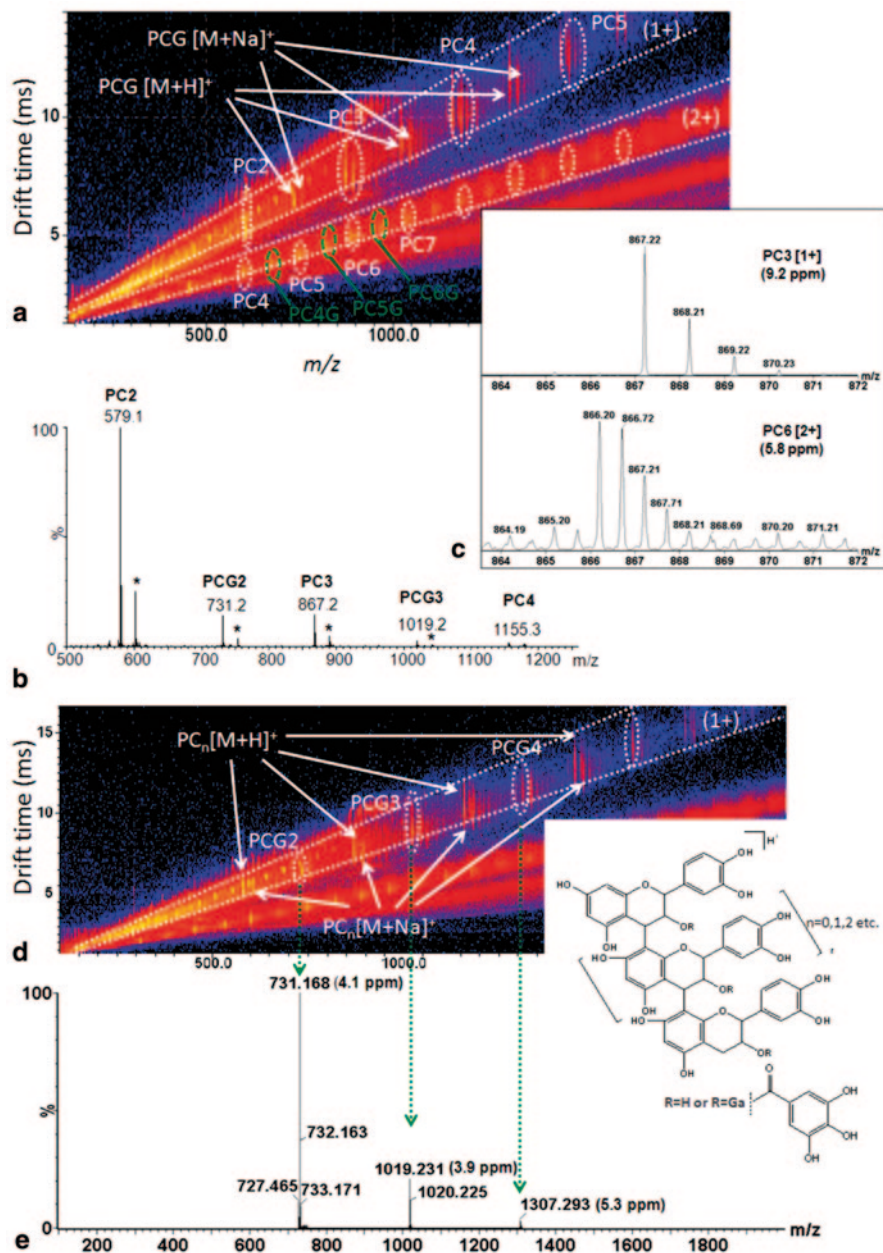


Fig. 2.2 TWIMS-MS analysis of grape seed proanthocyanidins. **a** TWIMS-MS driftscope image (drift time versus m/z) of a grape seed extract. PC ions separate in the TWIMS cell into charge groups labeled with $(+1)$ and $(+2)$ representing singly protonated and doubly protonated ions. **b** ESI mass spectrum of grape seed proanthocyanidins (depicted is range from m/z 500–1,450). The asterisk indicates the sodium adduct, $[M+Na]^+$, of the respective protonated molecular ion,

In the image depicted in Fig. 2.2d, arrows mark the singly protonated molecular ion clusters of procyanidins at m/z 579, 867, 1,155, and 1,443 corresponding to procyanidins with increasing degree of polymerization, namely DP2, DP3, DP4, and DP5. The sodiated molecular ions $[M+Na]^+$ are marked as well. The procyanidin monogallate ion clusters are encircled in this plot. Extraction of selected ion clusters allows the detailed analysis of ion signals that belong to a distinct proanthocyanidin series. For instance, the ion clusters of the procyanidin monogallates (PCG) were extracted and the respective mass spectrum of the extracted ion clusters is shown in Fig. 2.2b. The spectrum shows only the ion signals of procyanidin monogallates with DP2 (MH^+ , m/z 731.17), DP3 (MH^+ , m/z 1,019.23), and DP4 (MH^+ , m/z 1,307.29).

Although the present TWIMS–MS spectra were obtained in the positive ionization mode, proanthocyanidins give information-rich TWIMS–MS plots in the negative mode. Matrix-assisted laser desorption ionization (MALDI)–MS analysis has been described as a powerful approach for the characterization of PC mixtures [28, 30]. Therefore, it would be interesting to see if the combination of MALDI with TWIMS–MS would further advance the analysis of these highly complex biopolymer mixtures. The above example attempts to demonstrate some of the features that TWIMS–MS offers as an analytical platform for the in-depth interrogation of proanthocyanidin preparations. We anticipate that TWIMS–MS will emerge as measurement technology for the comprehensive analysis of other biopolymers and bio-inspired plastics as well.

Experimental Details The instrument was operated in positive ionization mode with an ESI capillary voltage of 2.75 kV and a sampling cone voltage of 30 V. The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas flow rate, 500 L/h; and cone gas flow rate, 40.0 L/h. Ion-mobility separation conditions included: ion-mobility gas flow rate, 75.30 mL/min, IMS wave velocity, 542 m/s, and wave height, 40.0 V. Argon was used as collision gas in the trap and transfer cells, while nitrogen (N_2) was used as IMS cell gas. Data acquisition was carried out using Waters MassLynx (V4.1), and for IMS data processing DriftScope software (V 2.1, Waters) was used. Positive ion mass spectra were acquired in the resolution mode over a mass range of 100–2,500 m/z using continuum mode setting. Mass calibration in positive mode was performed by infusing sodium iodide solution (2 $\mu\text{g}/\mu\text{L}$, 1:1 (v/v) water:2-propanol).

$[M+H]^+$. **c** The inset shows the extracted mass spectra of the singly protonated ions of procyanidin trimers, PC3, (upper mass spectrum) and the doubly protonated ions of procyanidin hexamers, PC6 (lower mass spectrum). Note the baseline-resolved isotope cluster for the doubly protonated PC6 ions; TWIMS–MS enables the gas-phase separation of the singly protonated ions from the doubly protonated PC oligomers avoiding overlapping of the isotope clusters on the m/z scale. **d** Distinct ion clusters can be individually extracted and exported to display the respective mass spectra. The extracted mass spectrum depicting singly protonated procyanidin (PC_n) oligomers with DP 2–4 is shown. Sodiated proanthocyanidin ion $[M+Na]^+$ has been annotated as well. Procyanidin monogallate (PCG) ion clusters are seen in between procyanidin clusters. **e** Extracted ion signals for procyanidin monogallates

2.4 Tandem Mass Spectrometry Approaches for the Structural Analysis of Plant Phenolics Using Dried Spot Analysis in Combination with TWIMS–MS

The need for high-throughput techniques for the analysis of dietary supplements and active ingredients encouraged us to explore the combination of dried spot analysis using thin-layer chromatography (TLC) plates in combination with ESI–TWIMS–MS. A combination of TLC, desorption electrospray ionization, and TWIMS–MS has been described previously for the direct analysis of pharmaceutical formulations [34]. The combination of ion-mobility separation with MS allows gas-phase ions to be separated by their mobility and then to be analyzed according to their mass-to-charge ratio in the TOF analyzer. Analysis specificity is further increased by combining ion-mobility separation with collision-induced fragmentation in the transfer region of the Synapt G2 instrument, thus, enabling the extraction of structural information and high resolution accurate mass measurements in one experiment. The combination of TLC-based spot analyses and ESI–TWIMS–MS resembles a multidimensional separation approach that results in high-content mass spectral information for the analytes of interest. Here, we describe the application of TWIMS–MS with tandem mass spectrometry for the analysis of bioactive flavonoids in dietary supplements, namely rutin (quercetin-3-O-rutinoside, $C_{27}H_{30}O_{16}$, M_{mono} 610.1534 Da) and hesperidin (hesperitin-7-O-rutinoside, $C_{28}H_{34}O_{15}$, M_{mono} 610.1898 Da).

Dietary supplements were extracted with methanol and the extracts were spotted on cellulose TLC plates. A CAMAG TLC MS interface combined with an LC system was used for eluting the analytes from the TLC plate and subsequent infusion into the mass spectrometer. Under the experimental conditions used, this approach enabled the interrogation of dietary supplement spots for a time period of approximately 1.5 min. A typical total ion response is shown in Fig. 2.3a. By extracting all ion signals at the plateau of the total ion chromatogram, a 3D driftscope image is generated (Fig. 2.3b). Note the relatively broad ion distribution at m/z 611 indicating insufficient resolution to separate the protonated ions of hesperidin and rutin under the TWIMS conditions used. However, two well-separated ion signal distributions were observed for the sodiated ions, $[M+Na]^+$, of those two flavonoid glycosides (m/z 633). The selected ion signals with m/z 633 show better signal-to-noise ratios than the protonated molecules $[M+H]^+$ at m/z 611 (Fig. 2.3d, e). Under the TWIMS conditions used, hesperidin ($[M+Na]^+$, m/z 633.18) and rutin

measured under the current conditions (m/z range 100–1,200 Da). Note the broad ion assemblies at m/z 611 and the two ion distributions with similar m/z values at m/z 633 but clearly different drift time distributions. **d** and **e** Comparison of drift time distributions of ion signals observed for two over-the-counter dietary supplements that contain both flavonoid diglycosides, hesperidin ($[M+Na]$ m/z 633.18, dt 5.21 ms), and rutin ($[M+Na]$ m/z 633.14, dt 5.97 ms). Drift time distributions were obtained by selecting the ions at m/z 633 with the quadrupole device Q1 followed by gas-phase separation of the ions in the TWIMS cell. In **e**, the mass spectrum has a different m/z scale than in **d** to illustrate that the ion signals for both flavonoid diglycosides ($[M+Na]^+$) are observable at the m/z scale

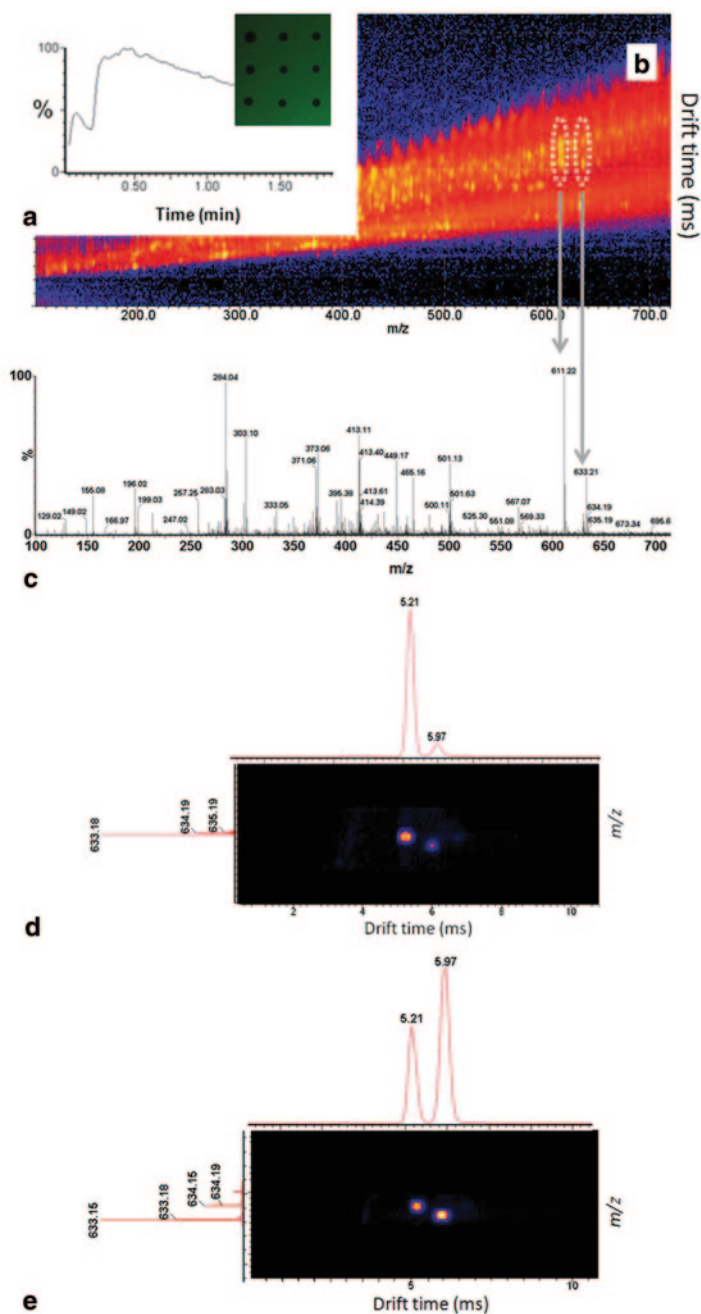


Fig. 2.3 Dried spot analysis of phytochemicals in dietary supplements with electrospray ionization TWIMS-MS. **a** Total ion chromatogram of a dietary supplement spotted on cellulose TLC plate, extracted from the plate, and infused into the mass spectrometer. **b** Driftscope image (drift time versus m/z) and **c** the integrated mass spectrum over the entire range of drift time distributions

($[M+Na]^+$, m/z 633.14) had drift time distributions centered around 5.21 and 5.97 ms, respectively.

In order to obtain structural information, the ions at m/z 633 were subjected to tandem mass spectrometry using the transfer region of the TriWaveTM device. Transfer fragmentation was conducted by selecting the ions at m/z 633 using the quadrupole device. Ions were then separated in the T-wave ion-mobility cell and subsequently subjected to collision-induced fragmentation in the transfer region (Fig. 2.4a). Applying elevated collision energy to the transfer device causes fragmentation of the mobility-separated precursor ions. Because the fragment ions preserve their velocity of the precursor ion, the fragment ions align with the drift times of the precursor ions. The ions at m/z 633 were selected in the quadrupole region, separated in the T-wave cell, and subsequently fragmented by collisions in the transfer region. The integrated fragment ion spectrum is shown in Fig. 2.4b. In Figs. 2.5 and 2.6, the fragment ion mass spectra of the sodiated molecular ions of hesperidin and rutin are shown, respectively. The time-aligned and compound-specific ions were extracted for each of the flavonoid glycosides separately. Because transfer dissociation experiments were conducted using sodiated precursor ions, the fragment ions are sodiated as well. Fragment ions of the rutinoside moiety dominated the spectrum for both species. The observed fragment ions are indicated in the schematic presentation of the two flavonoid glycoside structures.

Experimental Details The analytes were extracted using a CAMAG TLC interface from cellulose TLC plates using 75% acetonitrile/25% water. A Shimadzu LC-10AD pump was used for solvent delivery. The flow rate was 0.1 mL/min. Mass spectral experiments were performed using a Waters Synapt G2 HDMS mass spectrometer (Manchester, UK) equipped with TWIMS. Mass spectra were acquired in positive mode. The instrument was operated in the resolution mode with a capillary voltage of 3.0 kV and a sampling cone voltage of 30.0 V. The other conditions comprise the following: extraction cone voltage, 4.1 V; ion source temperature, 80 °C; desolvation temperature, 250 °C; desolvation gas flow rate, 500 L/h; and cone gas flow rate, 5.0 L/h. Ion-mobility separation conditions included ion-mobility gas flow rate; 90 mL/min, wave velocity ramping from 480 to 556 m/s; and wave height, 40.0 V. Argon was used as collision gas on the trap and transfer cells, while nitrogen (N_2) was used as IMS cell gas. Data acquisitions were carried out using Waters MassLynx (v4.1). IMS–MS data were processed with DriftScope software (v2.1, Waters). All analyses were conducted in the positive ionization mode. Mass spectra were acquired over the mass range of 50–1,200 m/z in continuum mode. A 0.1 ng/ μ L solution of leucine enkephaline ($[M+H]^+$ 556.2771) was infused at 5 μ L/min as the reference mass (lock mass) for accurate mass measurements. Mass calibration in positive mode was performed by infusing sodium formate (5 mM, prepared in 1:1 (v/v) $CH_3CN:H_2O$).

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