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Comprehensive two-dimensional gas chromatography, retention indices and time-of-flight mass spectra of flavonoids and chalcones

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ABSTRACT

The applicability of comprehensive two-dimensional gas chromatography (GC × GC) for flavonoids analysis was investigated by separation and identification of flavonoids in standards, and a complex matrix natural sample. The modulation temperature was optimized to achieve the best separation and signal enhancement. The separation pattern of trimethylsilyl (TMS) derivatives of flavonoids was compared on two complementary column sets. Whilst the BPX5/BPX50 (NP/P) column set offers better overall separation, BPX50/BPX5 (P/NP) provides better peak shape and sensitivity. Comparison of the identification power of GC × GC-TOFMS against both the NIST05 MS library and a laboratory (created in-house) TOFMS library was carried out on a flavonoid mixture. The basic retention index information on high-performance capillary columns with a non-polar stationary phase was established and database of mass spectra of trimethylsilyl derivatives of flavonoids was compiled. TOFMS coupled to GC × GC enabled satisfactory identification of flavonoids in complex matrix samples at their LOD over a range of 0.5–10 μg/mL. Detection of all compounds was based on full-scan mass spectra and for each compound a characteristic ion was chosen for further quantification. This study shows that GC × GC-TOFMS yields high specificity for flavonoids derived from real natural samples, dark chocolate, propolis, and chrysanthemum.

1. Introduction

Phenolic compounds are one of the most important, numerous and ubiquitous groups of compounds in the plant kingdom. Although, there are more than 8000 different known structures [1], a basic aromatic ring skeleton with one or more hydroxyl groups is a common structural feature, including for instance, simple phenols, phenolic acids, coumarins, flavonoids, stibenes, and tannins [2]. Flavonoids, as the largest ubiquitous group of plant secondary metabolites, make up over half the total phenolics distributed in various foods and medicinal plants. Flavonoids are a group of structurally related compounds with a chromane-type skeleton, with a phenyl substituent in the C_2 or C_3 position. More than 4000 known flavonoids comprise 12 subclasses [3], mainly subdivided into flavone, flavonol, flavanone, flavanol, isoflavone, catechin, chalcone, anthocyanin and so on [4].

Methods for identification of flavonoids are of interest both because of the widespread occurrence of these compounds in different natural products, and their reported potential health benefits, such as antioxidant, anticarcinogenic, antiarteriosclerotic, anti-inflammatory, antimicrobial, antiallergic, and antiaging activities [5–11]. Their potential role in protection against cancer and coronary heart diseases [12] is reported.

In principle, a wide range of analytical methods can be used to determine flavonoids in natural compounds, but it is important to bear in mind that the complexity of the matrix, which generally contains these compounds, makes mandatory the use of separative techniques with high resolving power. The most used techniques have been chromatographic techniques (TLC, CE, HPLC, GC), combined with spectroscopic methods (UV, IR, MS, NMR, NIR) [2]. Currently, the first option for flavonoids analysis is typically HPLC using reversed-phase (RP) methods coupled to DAD or mass spectral detection [13–16]. HPLC-MS, combining efficient separation capacity and structural characterization of MS, is successfully used for natural samples [3,17].

Although HPLC remains the most dominant separation technique for flavonoids, and HPLC can detect flavonoids across one, or more subclass in one run, natural products and mixed diet may contain several or even all subclasses [18]. It is a challenge for single column HPLC, with limited peak capacity to achieve quantitative separation of all flavonoids from each other, and from co-extracted

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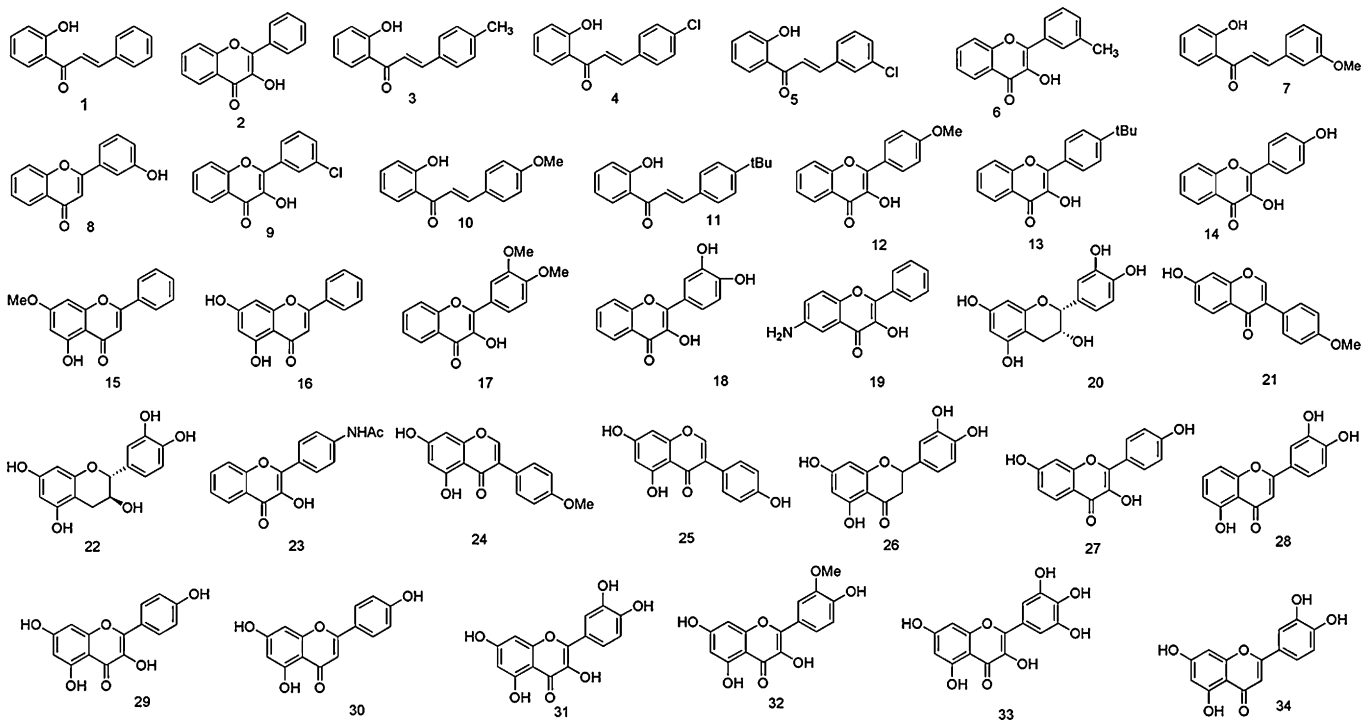

Fig. 1. Structures of flavonoids used in this study. See Table 1 for compound names.

chemical contaminants. This may compromise correct MS identification.

Gas chromatography (GC) with derivatization has been historically used for flavonoids analysis, especially before MS was available with HPLC. Most flavonoids and their metabolites are relatively polar, containing one or more functional groups (–OH, –COOH). Derivatization techniques provide enhanced gas phase volatility and stability for such polar and low volatility constituents. Normally, trimethylsilyl (TMS) derivatives of flavonoids are prepared to decrease polarity and to increase volatility and thermal stability of flavonoids, prior to GC analysis. TMS derivatives of flavonoids are stable, and give informative fragmentation ions under positive electron ionization (EI). The successful separation of TMS derivatives of flavonoids by GC followed by their mass spectrometric identification (GC–MS) has been reported by several researchers [19–21]. The ease of preparation and their excellent GC properties has led to an increased use of trimethylsilyl (TMS) derivatives for polar molecules for many compounds [22–26].

Comprehensive two-dimensional gas chromatography (GC × GC) has proved to offer significant advantages over conventional gas chromatography (1D GC) in terms of greatly increased peak capacity (separation power) and signal-to-noise ratio enhancement, and it also provides unique structured chromatograms when structurally related classes of substances (analogues, congeners, isomers) are analysed.

Briefly, GC × GC separation is based on two columns of distinctly different separation mechanisms, with a suitable interface (called a modulator) located near the coupling of the two columns. Further fractionation of the primary (first column, ^{1}D) separation, occurs on the short second (^{2}D) column, while preserving the previously achieved separation. Through use of a cryofocussing modulator, modulated peaks exhibit increased mass concentration due to the narrow focussed band of the chromatographic peak. Of signal importance to the analyst is the greater peak resolution achieved by GC × GC, effectively within the same time period as a conventional GC analysis, due to fast GC separation on the second column, i.e. within about 3–6 s. These very sharp peaks must be adequately detected, and identified, and for reliable area measurement about ten data points per peak are required [27]. This greater separation power means that many compounds are now completely separated, and this aids the correct MS identification of compounds separated in the GC × GC experiment.

Applications of GC × GC including petrochemicals [28], essential oils [29], amino acids [30], pesticides [31], food analysis [32], and forensic applications [33], amongst others.

The present study investigates the applicability of GC × GC coupled to both flame ionization (GC × GC–FID) and time-of-flight mass spectrometry detectors (GC × GC-TOFMS) for analysis of a wide range of flavonoids including flavones, flavonols, isoflavones, flavanonols, chalcones, and flavan-3-ols comprising a variety of substitution patterns and different numbers of derivatisable hydroxy groups. Selected flavonoid-rich complex natural materials are also analysed, with liquid/liquid extraction of the free flavonoids fraction, and derivatization with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) performed prior to analysis. Comparison between conventional GC and GC × GC, in terms of separation efficiency, sensitivity and identification power of TOFMS detection, is reported. A mass spectral compilation, a 10-peak MS ion abundance table, and retention indices are provided for all the compounds studied. This study illustrates the potential of GC × GC-TOFMS for flavonoids analysis, and detailed analysis of the complex profiles.

2. Experimental

2.1. Reagents

Analytical-reagent grade methanol and ethanol were purchased from Ajax Finechem Pty. Ltd. (Taren Point, Australia). HCl was from Merck (Darmstadt, Germany). Ultra pure water was supplied by a Milli-Q water purifier system from Millipore (Bedford, MA, USA). The derivatizing reagent was *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and was obtained from Supelco (Bellefonte, PA, USA) and maintained