



A brief review on emerging analytical techniques for essential oil quantification

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Abstract

Essential oils and their constituents have been utilized over centuries for their flavor, aroma, and medicinal applications. Quality assurance of essential oils is need to be focused because it greatly affects their therapeutic values. This review encompasses an overview of emerging analytical approaches used for the characterization of essential oils. Conventional approaches including GC-FID and GC-MS provide quantitative and qualitative analysis, respectively. Enantio-selective GC allows analysis of chiral constituents of essential oils. A useful alternative to GC is HPLC as it gives both qualitative and quantitative information. Hyphenated techniques such as LC-MS-MS provide complementary information about structural profile of essential oils. Multidimensional chromatographic methods have been established to overcome co-elution issues as they provide better resolution. ¹³C-NMR spectroscopy is preferably used for quantification of stereoisomers and heat sensitive compounds. The range of information obtained from analysis of essential oils enables the application of chemometrics.

Keywords: volatile oil, multidimensional chromatography, chemometrics, chiral, essential oil, quantification

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1. Introduction

Since the middle ages, essential oils have been widely employed for anti-microbial, anti-parasitical, insecticidal and medicinal applications, especially in the field of sanitary, folk medicines, pharmaceuticals, cosmetics, flavor, and fragrance industries. Major advantage of medicinal uses of essential oils is that they generally do not pose long-term genotoxic threats. In addition, some of them show very pronounced anti-mutagenic capacity that can be well associated with anti-tumor activity. Naturally, these oils play a vital role in plant protection [1]. The composition of volatile oils, because of physicochemical limitations of volatility, is constrained to low molecular weight hydrophobic products belonging to the classes of terpenes, phenyl-propanoids, benzenoids, and non-terpene aliphatic compounds. Terpenes can be considered the structurally most diverse class of natural plant products resulting from the repetitive fusion of isoprene subunits. Factors including climate, genetic traits, cultivation techniques, and soil have a large impact on the composition of essential oil in various plant species. Moreover, the profile of essential oils is also influenced by extraction procedure used. Essential oils are generally obtained by steam, hydro, or dry distillation or by expression (unheated

mechanical process applied to citrus fruits) of a plant or plant part. Currently, over 3000 volatile oils are known, of which 300 are commercially viable, particularly in the sanitary, agronomic, perfumery, pharmaceutical, and food industries. The tremendously diverse and complex nature of this group of natural products has become more comprehensible by researchers with the discovery and lucubration of millions of essential oils and its constituent compounds with the passage of time [2]. The storage conditions, product authenticity and shelf life are ensured by maintaining proper quality control [3]. Essential oils are characterized by using analysis technique which can elucidate its chemical composition such as gas chromatography along with mass spectrometry (GC-MS) is a frequently used technique. In recent years, several research publications have provided results of GC-MS and GC-FID analysis of essential oils extracted from various plant species. Classes of compounds that are constituents of essential oils may also be characterized additionally or alternatively by HPLC. Diode array and mass spectrometer are often used as detectors. Compounds which are intrinsically mixtures of different constituents e.g. essential oils, can be analyzed by various recent techniques like multi-dimensional LC, GC and GC-LC [4]. Another important characterization technique used for essential oil analysis is NMR spectroscopy which is also used to bolster results obtained

from GC-MS. Such analysis has helped increase knowledge and understanding of essential oil constituents and their properties over decades. Moreover, novel analysis techniques have provided further information about essential oils. In view of these reasons, a comprehensive review of recent developments in analytical techniques for the characterization of essential oils seems beneficial.

2. Chromatographic Techniques

2.1. Gas Chromatography (GC)

The diversity and complexity of molecular structures that can be found as components of essential oil require high resolution chromatographic procedures in order to quantify the contributors. GC is a most common analytical technique used in essential oil analysis. Polarity and volatility of essential oil components made the CGC (capillary gas chromatography) the technique of choice. For characterization, the analytes are usually separated on capillary columns with various stationary phases, including polar polyethyleneglycol (Carbowax®, HP-20, etc.) and non-polar methyl-phenyl-polysiloxanes (DB-5, CPSil-5, and HP-5) and methyl polysiloxanes (DB-1, HP-1, OV-101, etc.). Compounds are identified by information obtained from detector (retention time locking, Kovats indices and retention indices) or spectral representation of mass spectrometric results and also by combination of these two techniques [5]. The GC analysis of volatile plant oil samples are best carried out by using detectors which are most commonly used for mass spectrometry. The eluate coming out of gas chromatographic column consists of constituents which are converted into their respective ionic forms by either CI (chemical ionization) or EI (electron impact) and then these charged molecules or molecular fragments are separated on the basis of mass-to-charge (m/z) ratio and then enters quadrupole mass filter and quadrupole ion trap. The information regarding retention time of constituent compound is gained from total ion chromatograms and the mass spectrums provide fragmentation pattern of characteristic ions. A mass spectrometer of considerable sensitivity has detection limits in picogram range for the full scan mode (wide range molecular scanning) while in selected ion monitoring mode (SIM) for a particular compound, it could gain levels even lower i.e. femtogram range [6]. For the analysis of volatile compounds, Fourier transform infrared spectroscopy (FTIR) is an alternative of MS to be used with GC (GC-FT-IR) as its detector. Isomers with similar EI mass spectra can be differentiated by using this spectroscopic technique [6]. Fourier transform infrared spectroscopy has the capacity to distinguish between very similar isomers of a compound and also to provide information about whole (unfragmented) molecule. Major drawback of using this spectroscopic technique is the unavailability of commercial IR-spectra libraries which lead

to problems related to quantification and interpretation of data obtained. Similar issues are being faced for the utilization of GC-AES (gas chromatography atomic emission spectroscopy) and GC-UV (gas chromatography ultraviolet spectroscopy) as characterization techniques. This led to lack of importance given to these techniques in essential oil research. However, gas chromatography with isotope ratio mass spectrometry (GC-IRMS) by means of a combustion interface has emerged as another significant analytical tool for essential oil characterization. The accessibility to the quantification of ratios of stable carbon isotopes $^{13}\text{C}/^{12}\text{C}$ was made possible by commercially available GC-C-IRMS. A number of research investigations have reported information obtained through this technique [7-9].

2.2. Enantio-Selective Gas Chromatography

The development of cyclodextrin based stable chiral phases has permitted detailed study of enantiomeric profile of essential oil components [10-11]. Such type of analysis has been employed for the detection of contaminants, determination of oil composition for quality control, and to investigate the basic mechanisms involved in terpenoids biosynthesis [12]. Many essential oils have been already tested with enantio-selective gas chromatography using discrete chiral stationary phases, but it turned out that effective separation cannot be achieved on a single chiral column. A study developed columns that deal with chiral separations of specific problems, noting that some phases preferentially resolve certain enantiomers [13]. Until now more than 100 stationary phases with immobilized chiral selectors have been used, there is no universal chiral selector with high potential for separation of enantiomers [5]. Two dimensional approach is often needed for the chiral separation of complex matrix compounds including essential oils [14]. Innovations in enantio-selective gas chromatography are associated not only with the continuous development and use of various chiral stationary phases, but also with the development of enantioselective analytical methods. Some of the most recently used techniques for analyzing essential oil samples are: ES-GC-MS (enantioselective gas chromatography-mass spectrometry) and fast ES-GC-MS [15]. Isotopic ratio mass spectrometry (IRMS) hyphenated with ES-GC is currently the most accessible approach to prove the authenticity of the sample as the most effective tool for detecting essential oils containing optically active constituents [9].

2.3. Gas Chromatography-Olfactometry

Gas chromatography-olfactometry (GC-O) is a well-known standard method used for the assessment of odorant constituents found in complex mixtures. The technique is based on the correlation among

chromatographic peaks of eluted components simultaneously perceived by the two detectors, one of those being human olfactory system. Gas chromatography-olfactometry has been extensively utilized in the analysis of essential oils for many years, particularly in *Citrus* sp. by using different assessment protocols [16-18]. GC-O results provide information about the absence or presence of odor in essential oils, determine the quality of perceived odor, measure the duration of odor activity, and quantify the intensity of specific odorant that may have a crucial role in the utilization of such compounds in flavor and fragrance industries. GC-O is also being used to trace the odor-active areas in the chromatogram and to generate an odor profile for the oil sample [19].

2.4. High Performance Liquid Chromatography

HPLC-UV can be a complementary or even alternative technique for analyzing volatile oil due to its versatility, selectivity, and sensitivity [20]. HPLC is usually the method of choice for analyzing the less volatile components of essential oils [21]. The application of HPLC-MS provides excellent information on the content and nature of complex matrix components [22]. In spite of above mentioned benefits of high performance liquid chromatography, only a relatively small number of reports on HPLC analysis of essential oils are found in literature [23]. A study used the HPLC-UV (reversed phase mode) for the quantification of *Ribes nigrum* L. essential oil [24]. Another study developed a fast HPLC system for the analysis heterocyclic compounds of oxygen in citrus oil. Five different oils have been characterized by reversed phase HPLC using photodiode array detector (DAD) [3]. Heterocyclic compounds of oxygen including psoralens, polymethoxylated flavones, and coumarins found in essential oil of citrus (bitter orange, lime, lemon, grape fruit, and sweet orange) have been quantified using HPLC-MS system equipped with atmospheric pressure chemical ionization (APCI) source [22-25]. Application of LC-APCI-MS (liquid chromatography-atmospheric ionization mass spectrometry) equipped with reversed phase C-30 column, in the quantification of carotenoid esters found in essential oil of mandarin has also been reported. Detection of appropriate amounts of cryptoxanthin esters indicated mandarin essential oil a potential source of precursors of vitamin A [26]. Two alternative techniques including capillary liquid chromatography (CLC) and micellar-electrokinetic chromatography (MEKC) have been investigated for the determination of selected volatile constituents in orange essential oil (cold-pressed). Capillary liquid chromatography permitted the quantification of terpenes including mircene, α -pinene, limonene, and terpene derivatives such as carvone, linalool, and citral while, in micellar-electrokinetic chromatography mircene, limonene, citral, carvone, and linalool were detected [27]. GC, GC-

MS, ^{13}C -NMR have been used in conjunction with high resolution LC-MS and LC-MS-MS, to analyze the chemical profile of *Achillea millefolium* and *Achillea crithmifolia* essential oils [28].

3. Multi-Dimensional Chromatographic Techniques

Complex samples such as volatile oils, need analytical protocols with extremely high resolving power to ensure complete analysis of sample compounds. Multidimensional chromatography is a technique that provides higher resolution [29].

3.1. Multidimensional Gas Chromatography

Despite significant advances in instrumental technique, the complete detection of essential oil constituents remains problematic. Due to complexity of the sample compositions, a single capillary column is unable to completely separate interested components within an acceptable time. The widespread peak overlap and structural similarity of many terpene compounds are often an obstacle to a reliable structural explanation of mass spectrometer [29]. Unambiguous identification of minor compounds is extremely difficult to accomplish when high concentration matrix components mask their presence in the sample [30]. Thus, in many circumstances, two dimensional gas chromatography (GC-GC) must be done. This hyphenated technique involves coupled columns of varying polarity and subjects test samples to two independent steps, characterized by the orthogonal selectivities [18]. Generally, the first column is a column with less polar stationary phase, which affects the dislocation of analytes along x-axis of the separation plane, while the second column is a column of polar stationary phase (column arrangement depends on the sample). Different compounds are subjected to different separation mechanisms, and there are no specific rules for the column phase type combinations as long as they offer orthogonal separations. Selected effluent fractions unresolved in first column are allowed to transfer to the second one, in a process known as heart-cutting [30-31]. The instrumentation includes modulator (transfer device), sandwiched between two dimensions, which represents the system key and enables the entire process. In the first multidimensional gas chromatography (MDGC) systems, the transfer of effluent fractions from the 1st to the 2nd dimension was attained by mechanical valves. This approach has been abandoned and substituted by a new interface that maintains pressure balance between two columns [32]. Subsequently, the system has been improved and widely applied in the analysis of enantiomeric volatile constituents of citrus essential oil. A study demonstrated a different and cost effective approach for transferring individual GC peaks to second column using solid phase micro-extraction device [33]. Another study developed a six

rotary mechanical valve as an interface that was later refined by the same researchers and used to determine enantiomeric compositions of citrus essential oils [34]. Considerable improvement in two dimensional gas chromatography occurred with the implementation of an entirely new form of two dimensional comprehensive gas chromatography (GC-GC), in 1991 [35]. Application of this hyphenated technique in the analysis of peppermint and spearmint essential oils showed a two-three fold enhancement in separation power over mono-dimensional gas chromatography [36]. Subsequent reports demonstrated the separation of tea tree, lavender, and vetiver essential oils which led to the steady development of GC-GC for analysis of fragrant compounds, and the technique is now widely employed [37]. Hyphenation of GC-GC with time of flight mass spectrometry (TOFMS) represents a three dimensional technology. This hyphenated technique was first applied for separation and characterization of lavender essential oils and has been further optimized to completely exploit three analytical dimensions [38]. In a study, 394 compounds have been characterized from *Pogostemon cablin* essential oil using this technique (GC-GC-TOFMS) [39]. The high cost of GC-GC-TOFMS, led to the investigations of suitability of qMS (quadrupole mass spectrometry) for GC-GC-qMS system. The technique was first applied for the characterization of essential oil of *Pelargonium graveolens*. Comparatively, the slow data acquisition speed may not be appropriate for quantitative analysis, but enhanced separation quality over mono-dimensional techniques is apparent [40].

3.2. Multidimensional Liquid Chromatography

Two dimensional liquid chromatography is a novel technique which couples two independent liquid chromatographic (LC) separation systems having orthogonal selectivities [41]. The application of two dimensional reverse- and normal-phase modes can lead to the separation of complex natural materials containing uncharged compounds of comparable size, different in hydrophobicity and polarity [29]. Several manuscripts relating to the applications of two dimensional liquid chromatography for essential oils analysis have been published [29-42-43]. A study analyzed heterocyclic oxygen compounds of cold-pressed *Citrus limon* (L.) oil using comprehensive two dimensional liquid chromatography (2D-LC) that is based on the application of microbore silica column operated in 1st dimension in normal-phase mode, and a C18 column (monolithic type) operated in 2nd dimension in reversed-phase mode [29]. Another study described the capacity of technique in analyzing and identifying the natural carotenoid profile of an extremely complex matrix such as blood orange essential oil. Fourty different carotenoids have been identified in orange oil including free carotenoids (3), carotenoid monoesters (16), and carotenoid diesters (21),

using LC-LCDAD/APCIMS without any pretreatment [42]. Same researchers applied two different comprehensive HPLC techniques to analyze the carotenoid profile of mandarin volatile oil. A monolithic C18 column was coupled with silica microbor column to examine saponified extract of mandarin, however, the coupling of monolithic C18 column with cyano microbore column was used to examine the whole mandarin volatile oil sample to analyze the composition of natural carotenoid esters. Detection was achieved by connecting mass spectrometer (MS) detector in parallel with photodiode array detector (DAD) operated with an APCI (atmospheric pressure chemical ionization interface) [43].

4. ¹³C-NMR Spectroscopy

The ¹³C-NMR analysis of essential oil components is based on a comparison of ¹³C-NMR spectra of oils with spectra of pure compounds recorded under ideal conditions. Karl-Heinz Kubeczka, a pioneer in the application of ¹³C-NMR in the analysis of essential oils, gave an overview of analytical methods applied in the characterization of oils including ¹³C-NMR [43]. He with Formacek published a book containing ¹³C-NMR spectra of sixty commercial oils used in cosmetic, pharmaceutical, food, folk medicine, and perfumery industries. These spectra helps in the authentication of commercial oils and the identification of individual constituents of the oil tested. ¹³C-NMR spectra are usually used in combination with GC-MS data [28-44-45]. However, advantage of this technique over GC-MS is the analysis of thermolabile compounds, stereoisomers, and other compounds which show poorly resolved spectral patterns [45-46]. A study determined the actual content of ascaridol (heat-sensitive compound) using ¹³C-NMR spectroscopy [47]. Gas chromatographic analysis provides lower amounts of ascaridol because of thermal degradation of ascaridol to isoascaridole. Various computational techniques have been presented for the identification of essential oil chemical constituents through the analysis of ¹³C-NMR data. Some of these rely on signal intensities and chemical shifts, while others consider multiplicities obtained from ¹³C-NMR DEPT spectra [48].

5. Chemometrics

Recent advances in chemometrics and statistical softwares have led to expand the horizons and scope of evidence extracted from traditional analysis of volatile oil fractions of plant [14]. Results obtained from spectroscopic analysis are followed by statistical treatments which are determined by test samples are usually preceded statistically by their chromatographic profiles determined by the abundance of a selected number of markers defined as variables. "multivariate analysis" is most frequently used method, particularly PCA (Principal Component Approach)

is applied for the description of differences within the samples characterized by number of variables in which variables explaining variability are combined in linear fashion [14]. A distinction between groups of samples with variables (MS fragments or individual constituents) of greatest statistical significance in distinguishing samples, can be made with the help of this method. This provides appreciable information on origin or chemotypification [5-49] as well as on differentiation between species at intra-genetic levels and/or the technological treatments [50].

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