

Critical Reviews in Food Science and Nutrition

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/bfsn20>

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Accepted author version posted online: 08 Mar 2013.

To cite this article: Karl Fraser , Scott J. Harrison , Geoff A. Lane , Don E. Otter , Yacine Hemar , Siew-Young Quek & Susanne Rasmussen (2013): Analysis of low molecular weight metabolites in tea using mass spectrometry-based analytical methods, Critical Reviews in Food Science and Nutrition, DOI:10.1080/10408398.2011.619670

To link to this article: <http://dx.doi.org/10.1080/10408398.2011.619670>

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Analysis of low molecular weight metabolites in tea using mass spectrometry-based analytical methods

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Abstract:

Tea is the second most consumed beverage in the world after water and there are numerous reported health benefits as a result of consuming tea, such as reducing the risk of cardiovascular disease and many types of cancer. Thus there is much interest in the chemical composition of teas, for example; defining components responsible for contributing to reported health benefits; defining quality characteristics such as product flavour; and monitoring for pesticide residues to

comply with food safety import/export requirements. Covered in this review are some of the latest developments in mass spectrometry-based analytical techniques for measuring and characterising low molecular weight components of tea, in particular primary and secondary metabolites. The methodology; more specifically the chromatography and detection mechanisms used in both targeted and non-targeted studies, and their main advantages and disadvantages are discussed. Finally, we comment on the latest techniques that are likely to have significant benefit to analysts in the future, not merely in the area of tea research, but in the analytical chemistry of low molecular weight compounds in general.

Keywords: Polyphenols; Electrospray ionisation; Alkaloids; UHPLC; GC-MS; LC-MS

INTRODUCTION

There are many instrumental techniques for analysing the composition of tea and the scope of this review is to cover specifically the mass spectrometric based methodologies used in detecting and measuring low molecular weight primary and secondary organic compounds/metabolites in tea. Mass spectrometric analyses have an advantage over many other instrumental techniques as they can provide useful characterising information as well as the ability to detect and measure a wide range of compounds, thus significantly increasing the amount of information that can be generated about a sample. The application of mass spectrometry (MS) has increased recently mainly due to the reduced cost of these instruments, but also because of their greater ease of use along with an increase in the types and combinations of mass analysers available. There is

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extensive literature on the determination of major secondary metabolites such as the polyphenolics and volatiles in teas, for which liquid chromatography (LC) or gas chromatography (GC) respectively predominate (Pripdeevech and Machan, 2011; Wang and Ho, 2009). Thus we have biased this review towards literature that involves the coupling of separation techniques such as LC and GC with MS, as this can significantly increase the amount of knowledge that can be gained from a sample.

Tea is a beverage originating from China that has been consumed for thousands of years. It is a product made from the leaf and bud of the plant *Camellia sinensis* and is the second most consumed beverage in the world after water (Cabrera et al., 2006). This makes tea a very important commodity for some developing countries in terms of employment and export earnings, for example Sri Lanka where tea production was responsible for 1.2% of gross domestic production in 2007 (De Costa, 2010). The worldwide production of tea in 2007 was almost 3.9 million tonnes with the majority of production occurring in China, followed by India, Kenya and Sri Lanka (Alkan et al., 2009) and the average per head consumption of tea is approximately 500 mL/day (Kuhnert, 2010).

Generally tea can be broadly classified according to its production method as either unfermented (green tea), semi-fermented (oolong tea), fully fermented (black tea) or post-fermented (pu-erh tea) (Zhao et al., 2006). Black tea is drunk worldwide but mostly in North America, Europe, and North Africa. Green tea is widely consumed in Japan, Taiwan, Korea, China, and Morocco while oolong tea is popular in China and Taiwan. Of tea production and consumption

worldwide, approximately 76-78% is black tea, 20-22% is green tea and less than 2% is oolong tea (Cabrera et al., 2006).

Tea is a rich source of polyphenolics, particularly flavanoids, which are effective antioxidants found throughout the plant kingdom (McKay and Blumberg, 2002). Flavanoids are phenol derivatives synthesised in substantial amounts (0.5-1.5%), with a wide diversity (more than 4000 identified) and are widely distributed among plants (Vinson et al., 1995). The main flavanoids present in green tea are catechins (flavan-3-ols) such as epigallocatechin-3-gallate (EGCG, Fig. 1), epigallocatechin (EGC), epicatechin (EC), and epicatechin-3-gallate (ECG). Figure 1 also shows a polymerised catechin theaflavin-3'-gallate which is a predominate component of black tea, along with other theaflavins and thearubigens (McKay and Blumberg, 2002). Other major theaflavins in black tea are theaflavin, theaflavin-3-gallate, and theaflavin-3,3'-gallate (Wang and Ho, 2009). Theaflavins and thearubigens are responsible for the characteristic colour and flavour of black tea (Graham, 1992). Other phenolics found in tea include the flavonols, principally quercetin (Fig. 1) and kaempferol and to a lesser extent myricetin, and they generally occur as mono, di and tri *O*-glycoside conjugates (Lin et al., 2008). Tea is an important source of gallic acid, a hydroxybenzoic acid. The amount of gallic acid in tea increases during the fermentation process of tea manufacturing owing to its liberation from catechin gallates. Chinese pu-erh teas contain the highest levels of gallic acid (approximately 15g/kg dry weight) compared to other types of tea (Lin et al., 1998). Tea contains caffeine which is present across an average concentration range of 20-40 mg/g (Lin et al., 2003), along with very small amounts of the other common methylxanthines, theobromine and theophylline. The amino acid theanine

(5-N-ethyl glutamine, (Fig. 1)) is unique to tea (Graham, 1992) and is present at many times the level of any other amino acid in the plant (Syu et al., 2008).

Tea Processing

After harvest, the leaves soon begin to wilt and oxidise if not dried quickly after picking. Green tea production is characterised by allowing a partial wilting step followed by a quick heating process to stop any post-harvest fermentation. This fermentation process is an auto-oxidation catalysed by the enzyme polyphenol oxidase present in the leaf and is extremely important for the production of both oolong and black tea. The enzymes convert the flavanols in the leaf into the polyphenolic compounds, such as the theaflavins and thearubigens, which contribute to giving black tea its dark colour. For oolong and black tea, the level of oxidation is monitored and stopped at the required predetermined stage by heating to deactivate the polyphenol oxidase. A schematic illustrating the steps involved in the production of the three major types of tea is shown in figure 2 (Balentine, 1992; Tomlins and Mashingaidze, 1997).

Pu-erh tea manufacturing is slightly different and is typically done via one of two methods. Raw pu-erh tea is traditionally processed by pressing large unoxidised leaves that are then fermented for several years at room temperature. Ripened pu-erh teas have the leaves post-fermented with microorganisms (such as *Aspergillus niger*) for several months under optimum conditions prior to being pressed (Chen et al., 2009).

Reported health benefits of drinking tea

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There have been many studies investigating the purported health benefits of consuming tea, particularly so over the last decade where people have generally become more aware of health issues and functional foods that may provide significant health benefits upon regular consumption. The health benefits related to tea appear to be linked to the polyphenols present, although the types and levels of polyphenols in the major tea types can vary significantly due to the different manufacturing processes. Numerous biological studies have concluded that EGCG is the most beneficial polyphenolic to human health. However, other phenolics such as gallic acid have also demonstrated significant health benefits (Arab and Liebeskind, 2010; Gupta et al., 2008; Lambert and Elias, 2010; Moon et al., 2007).

There is a vast amount of recent literature around claimed benefits of green tea consumption to human health and the list of reported beneficial effects are long and will only be mentioned in passing in this article. Several reviews (Cabrera et al., 2006; Chacko et al., 2010; Clement, 2009; Sinija and Mishra, 2008) on this area over the past few years have summarised evidence for health benefits such as; decreased risk of a wide range of cancers such as breast, prostate, liver, ovarian, gastro-intestinal, and lung cancer; decreased incidence of cardiovascular events and stroke; beneficial effects for patients with type II diabetes; increased plasma antioxidant levels for improved protection against reactive oxygen species; increased metabolism and anti-obesity effects.

The benefits of consuming oolong tea have not been as extensively studied as for those of the more common green and black teas, however, some reported health benefits include; increased metabolic rate and fat oxidation in men (Rumpler et al., 2001) and decreased obesity (He et al., 2009); decreased effects of dermatitis (Uehara et al., 2001); inhibition of the growth of human

stomach (Hibasami et al., 2000) and colon cancer cells (Hibasami et al., 2003); inhibition of allergic reactions (Sano et al., 1999) and aid in the control of diabetes (Hosoda et al., 2003).

Recent literature reviews into the beneficial effects of black tea consumption (Arts, 2008; Gupta et al., 2008; Ruxton, 2009) have stated that many of the same effects reported for green tea consumption can be obtained by consuming black tea such as; reduced risk of coronary heart disease and stroke; inhibition of lung, ovarian, breast, and renal cancer; improvements in cognitive function; and increased anti-oxidant levels to name a few.

While laboratory studies show great potential for positive health effects of tea consumption, the human clinical evidence is still limited and many of the published human studies state contrasting results, particularly in the area of cardiovascular disease and cancer. Future human studies must carefully define tea preparations, intake levels and sample populations to define the actual magnitude of health benefits and give firm conclusions about its bioactive value to humans.

The requirement for analytical methodologies

Tea is a crop of significant commercial value consumed by humans and as such common applications of analytical methods are principally for monitoring the quality and safety of the product (Le Gall et al., 2004; Ochiai et al., 2005). Methods for determining components and their concentrations have been developed for a multitude of reasons, such as to improve the understanding of how components contribute to flavour, shelf life, quality and colour, or to detect and quantify contaminants such as pesticides or mycotoxins. Even more so, they are

critical for understanding which component or class of compounds are responsible for potential health benefits that are associated with tea products and also their relation to processing methods. Analytical methodologies can be used in elucidating the mechanisms of action and to discover good predictive biomarkers. In addition, by improving our understanding of the biochemistry of tea, there is potential for using analytical technologies to authenticate products, for example by confirming the country of origin, tea type, and purity of the final product (Engelhardt, 2006).

Mass Spectrometry

Mass spectrometry is defined as a detection technique that uses the difference in mass/charge ratio (m/z) of ionised molecules to separate them from each other (de Hoffmann, 2000). Mass spectrometry is useful for quantification of molecules and determining their molecular weight, as well as giving chemical, and structural information about molecules and the history and principles of operation of mass spectrometers have been well described in the literature (de Hoffmann, 2000; Griffiths, 2008; Gross, 2004). A mass spectrometer generally consists of an ion source, a mass-selective analyser, and an ion detector (Steinmann and Ganzenra, 2011). Since mass spectrometers create and manipulate gas-phase ions, they are required to operate at high-vacuum and require extraction and acceleration ion optics to transfer ions from the source region into the mass analyser. The most common mass analysers currently available are the quadrupole (Q), ion-trap (IT) and time of flight (ToF), or combinations of these analysers such as IT-ToF, q-ToF, or triple-quadrupole (QqQ); however, these tandem MS instruments are more expensive than the single stage mass analysers (Allwood and Goodacre, 2010).

As MS systems require ions in the gas phase, the traditional coupling of chromatography and MS systems has been GC-MS, as the molecules are already in the gas phase after chromatography. The two main techniques used for charging molecules in GC-MS are electron impact ionisation (EI) and to a much lesser extent chemical ionisation (CI) (Kind and Fiehn, 2010). EI involves the molecules entering a ‘stream’ of electrons fired at 70 electron volts (eV) within a high vacuum where they become charged, generating a molecular ion and fragments in a reproducible and reliable manner related to the structure of the molecule (McLafferty and Turecek, 1993). This consistent fragmentation means that mass spectral fragmentation libraries can be created and searched and there are several commercial libraries available for use on any GC-MS instrument generating data at 70 eV. Generally, EI generates characteristic fragmentation patterns which can enable identification or at least provide evidence regarding chemical classification. However, with some classes of analytes, when the molecule fragments it does not generate a detectable molecular ion, causing problems when seeking confirmation of their identity, for example, within the class of long chain alkanes such as dodecane or tetradecane. CI is a soft ionisation technique for GC-MS involving the collision of analyte molecules with charged ‘reagent’ ions such as methane or ammonia contained in the ion source area, and subsequent charge transfer to the target molecule (Munson and Field, 1966). This charge transfer method generally provides only limited fragmentation and a more intense molecular ion, which enables better determination of the molecular weight of the target species.

The development of suitable ionisation sources for interfacing liquid chromatography with MS such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) has

enabled MS to be used to measure a much wider range of compounds, from low molecular weight sugars to high molecular weight intact peptides and proteins, not just volatiles or semi-volatiles as for GC-MS (Smith et al., 1990). These ionisation techniques use high voltage to charge the compound and large quantities of nitrogen and heat to aid in evaporation/desolvation of the mobile phase containing the compound (Gaskell, 1997) (although many ESI applications are not heated). Another ionisation mechanism is to fire an ionising beam at a surface to generate the ions via charge transfer. This technique can be used in applications such as direct electrospray ionisation (DESI) where an ESI probe is placed above a sample and the spray directed at the surface of the sample to generate ions from the surface. Another ionisation method which is generally used for higher molecular weight components is matrix assisted laser desorption ionisation (MALDI), where a compound/mixture is mixed with another ‘matrix’ compound such as dihydroxybenzoic acid and allowed to dry and form a thin layer of co-crystals (Zenobi and Knochenmuss, 1998). This layer is then irradiated with a high energy laser which, with the aid of the matrix, generates ions for detection. However, a major limitation of this technique is that it cannot be coupled directly with chromatographic systems. The ionisation techniques described above are known as soft ionisation and are capable of ionising the majority of molecules without causing fragmentation to generate what is known as the ‘molecular ion’; this enables a molecular weight of the component to be measured. By altering source and instrument settings however it is possible to get some fragmentation occurring. Some fragmentation in the source area can be achieved by increasing the acceleration of the ions through the partial vacuum region which causes collisions with other neutral gas phase molecules and some fragmentation. The generation of mostly molecular ions is very useful in

tandem MS systems as they can both measure the ‘molecular ion’ and then isolate and fragment the ion in a controlled collision cell to either generate structural information or confirmation of the analyte, or to measure concentrations very selectively and sensitively.

MODERN MS BASED CHROMATOGRAPHIC INSTRUMENTS USED IN THE ANALYSIS OF TEA

As mentioned earlier, the application of MS in tea research has increased in recent years. With LC- and GC-MS, the amount of data generated by these hyphenated techniques can significantly increase the amount of information that can be gained from a sample. For example figure 3 shows the use of concurrent detection by LC-UV and MS/MS for the selective detection and characterisation of a component in a crude green tea extract. The collection of multiple levels of MS data can aid in the discrimination between similar compounds and assist in compound classification and characterisation to a much greater degree than UV data alone. As shown, there are more components detectable in the MS total ion chromatogram (TIC) than in either of the two selected UV wavelengths (Fig. 3). By using the selectivity of the MS, it is possible to detect and confirm that the component monitored is the target of interest via the MS₂ and MS₃ spectral fingerprints, confirming the presence of gallocatechin 3-*O*-gallate.

GC separation is a mature technology and since the development of capillary GC columns has provided much higher chromatographic resolution (better separation) than the best LC columns. Gradual improvements continue to advance the technique as column manufacturers continue to increase the durability and temperature ranges of the phases; for example, the development of

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metal columns for high temperature analysis of triglycerides. Instrument manufacturers have developed improved systems to enable higher pressure ‘fast GC’, and over the last few years the technique of two dimensional (2-D) GC, where two chromatographic columns of differing selectivity’s are used together to resolve co-eluting components, has become more widely accepted.

The recent development of smaller particle sized LC columns (sub 2 μ m) providing much improved chromatographic resolution for LC, and improved hardware to pump liquids at very high pressures (ca 18,000 psi), has seen the resulting ultra high pressure liquid chromatography (UHPLC) begin to dominate as the LC method of choice. The smaller particles give a far greater number of theoretical plates and thus superior chromatographic resolution compared to the older 5 μ m particle size columns (Nguyen et al., 2006). Many vendors are now producing UHPLC columns of differing phases that can tolerate pressures greater than 11,000 psi (Table 1), and with the growing market new phases become available frequently. The analyst can now select shorter columns with equivalent resolution to the older 5 μ m columns (e.g. a 50 mm sub 2 μ m column has equivalent resolution to a 150 mm 5 μ m column) which enables shorter run times and thus increased throughput, or use a similar length column and gain considerable improvements in peak capacity and chromatographic resolution of complex mixtures. Another advantage of these sub 2 μ m columns is that the elution width of retained components is much narrower than with conventional columns (ca 3 seconds vs. 15-20 seconds), resulting in an increased signal to noise ratio and thus sensitivity. However, UHPLC does require fast scanning detectors to ensure enough data points are collected across the peak (Guillarme et al., 2010b).

Targeted vs non-targeted analyses

There are a variety of different analytical techniques to consider when developing an analysis and they all come with their own unique pros and cons. Often these techniques are biased towards a specific class of compound, for example using a photodiode array detector (PDA) to detect analytes will result in detecting only compounds that contain an appropriate ultra violet/visible light absorbing chromophore. Generally when performing quantitative studies on particular known compounds the analyst will choose a more specific targeted detection method over a non-targeted method, and while this will usually give more accurate and sensitive results, changes in concentrations of compounds that are not specifically monitored will not be detected. Secondly, quantification requires baseline separation of all target analytes and as such can result in long runtimes and low throughput. Thus fast, sensitive, less-biased detectors such as MS have become more popular as they become more available. Table 2 lists the chromatographic and MS-based detection techniques commonly used in the analysis of compounds found in tea and some common examples of the applications.

Non-targeted methods allow detection of a much wider array of components, although maybe not to the same analytical accuracy or sensitivity, but are still biased by the nature of the extraction, separation and detection systems used (Dettmer et al., 2007). For example, GC-MS can only be used with volatile compounds (or compounds that can be made volatile by derivatisation) that are able to withstand the high temperatures used in GC-MS without degrading. However if a derivatisation step is required, the complexity of the analysis increases as a) there are many

different options for derivatising compounds, generally all with their own bias towards particular classes of compounds, b) there can potentially be multiple derivative peaks for a single component, and c) the derivatives may be unstable and degrade before analysis, affecting quantification (Kopka, 2006). Another issue with non-targeted analysis is the volume of data to be processed and interpreted, although software packages for multiple chromatographic alignment, peak picking, library building, and application of multivariate statistics are now being produced by many companies (Neumann and Böcker, 2010).

Targeted analyses and techniques

GC-MS:

GC-MS is an important technique for quantification of volatiles and has been well utilised in studies of characteristic volatiles of tea (Schuh and Schieberle, 2006; Wang et al., 2001a; Wang et al., 2001b; Wang et al., 2000). In this section we review recent or significant examples of the application of targeted GC-MS to tea.

An example of an application of quantitative GC-MS is the study of Wang et al. (2001b) on the change in glycoside content of tea leaves during black tea manufacturing processes. Trifluoroacetate derivatives of the mono- and disaccharides were chromatographed separately on capillary columns coated with either DB-5 stationary phase (non-polar) or HP-50 (medium polarity) stationary phases to fully resolve the complex mixture of isomers. Aroma concentrates of the final black tea product were made by steam distillation. Extracts and derivatives were run through the GC-MS in scan mode to record full scan spectra for both component confirmation and quantification. They observed that total glycoside contents in the leaves decreased

throughout the manufacturing process, with a 3 fold decrease in concentration of disaccharides after the leaves have been exposed to the rolling process, consistent with cell rupturing and release of plant glycosidase enzymes. Many low molecular weight volatile alcohols such as benzyl alcohol, linalool, and geraniol were detected in the aroma concentrates, sourced from the hydrolysis of their respective glycosides during the manufacturing process. They suggest that the “character of black tea aroma is mainly determined by the composition of aglycon moieties in the fresh tea leaves”, and thus the hydrolysis of these glycosides are important to the formation of black tea aroma. Whilst they achieved good quantification of derivatised endogenous glycosides, the need to run extracts separately through different polarity chromatographic columns decreases sample throughput. However, the throughput of this method could be improved using recently developed 2-D GC techniques as samples would only need to be run once (Cortes et al., 2009).

More recently Schuh and Schieberle (2006) used GC-MS to quantify differences between tea leaves and infusions prepared from Darjeeling black tea. They identified 24 aroma compounds contributing to the tea aroma by a GC-odour port sniffing experiment, and then quantified these in both the infusion and tea leaves using stable isotope dilution assay with synthesized isotopically labelled standards. Quantification by GC-MS with isotopically labelled standards is a very accurate method as the standards can be spiked into extracts and will have virtually identical properties to the target compounds, although the major problem with this type of methodology is the availability of commercial isotopically labelled standards, or the time required to synthesize them. They observed that the level of geraniol was 32 times greater in the infusion compared to the leaves. Although it is known that glycosides of geraniol occur in green

tea leaves, the authors suggest it is unlikely that these are the precursors in the black tea product as any plant enzymes will have been deactivated by the final firing process of the fermented tea leaves, thus the precursors are still unknown for these increased levels.

Much of the recent quantitative literature utilising GC-MS for the analysis of tea is concerned with the detection and quantification of pesticides, due to import and export regulations setting maximum residue limits of pesticides (Kuang et al., 2010; Ochiai et al., 2005; Schurek et al., 2008; Steiniger et al., 2010) in food products. Although these compounds are not primary or secondary tea metabolites, it is an important area of recent MS based tea research (Huang et al., 2007) and the methodology used could be applicable to other low abundance metabolites. As tea is a complex matrix, most studies have concentrated on the extraction, purification and enrichment of the sample although several authors have examined advanced instrumental techniques to aid detection within these ‘dirty’ matrices and we highlight two of the more recent publications below.

Advanced instrumentation was utilised to develop a method to detect 36 pesticides in tea using SPME combined with GC × GC/ToF MS (Schurek et al., 2008). They were unable to satisfactorily chromatographically resolve all the target pesticides with one dimensional GC, but were able to resolve all target compounds with GC × GC, and achieved improved signal to noise ratios with the 2-D method. The method showed good sensitivity, mass accuracy and resolution, important factors in component confirmation and for identifying unknowns. It is worth noting that to collect the very narrow peaks of GC × GC (10-20 times narrower than conventional GC peaks) the detector acquisition rate had to be increased to 125 Hz to obtain enough data points, a

data collection rate that is not possible with common scanning detectors such as ion-traps or quadrupoles.

Steiniger et al. (2010) recently published a method for determining pesticide residues in green tea using GC-ITMS. They investigated the efficiency of using a modified QuEChERS (acronym for quick, easy, cheap, effective, rugged and safe) extraction and the improved selectivity and specificity of an ITMS to quantify 22 pesticides in tea extracts. A QuEChERS extraction is done by homogenising the sample and shaking with extraction reagent (which can be adjusted depending on the target compounds), centrifuging, and then passing through a solid phase cartridge before chromatography (Lehotay et al., 2005). The functionality of ITMS instruments to select a precursor ion and fragment into product ions provided more precise quantification and higher confidence in the identities of the pesticides. The downside of ITMS is that the number of components that can be sequentially scanned is limited by the cycle speed of the trap and the chromatographic peak width. Consequently, the analyst must ensure there are enough data points across the peak to enable accurate peak area measurements and quantification. For optimal results, the ITMS precursor ion parameters should be segmented which requires reliable chromatographic separation and retention times.

GC-MS continues to be a well utilised technique in the quantitative analysis of teas, and the further development of methodology and technology for 2D GC and faster scanning detectors such as q-ToF MS systems and QqQ will aid in future technical advances.

LC-MS and LC-MS-UV:

There is a wealth of publications on quantitative studies of tea using LC-MS and LC-MS combined with standard online detectors such as UV and fluorescence (Chen et al., 2011; Del Rio et al., 2004; Dou et al., 2007; Guillarme et al., 2010a; Sultana et al., 2008). When using dual detection systems the MS data are often used for collecting qualitative information while the UV/fluorescent data are used for quantification. In this section we review recent or significant examples of the application of targeted LC-MS to tea.

Del Rio et al. (2004) published one of the more highly cited publications using LC-MS identified and quantified phenolics and purine alkaloids in tea. The authors used a slow reversed phase gradient on a long (250 × 4.6 mm) C12 column, splitting the column effluent so only 20% of the flow entered the ESI source. Samples were run in both +ve and –ve mode to enable the detection of both the phenolics (-ve) and purine alkaloids (+ve) such as caffeine and theobromine. The ITMS collected MS/MS information was used to aid in compound elucidation, and data collected simultaneously by PDA detection were used for quantification. The concentrations of the phenolics in green and black tea samples were then compared. Green tea phenolics belonged predominantly to the flavan-3-ols class, while black tea phenolics were predominately the condensation products of the flavan-3-ols, the thearubigins. Although technically advanced for the time, this methodology has generally been improved and superseded by UHPLC and faster scanning and more sensitive MS instruments.

Sultana et al. (2008) performed a quantitative analysis of flavonoids from tea samples of different origins by LC-PDA and LC-MS. A thorough evaluation of extraction methods was performed and they determined that the chemical composition could be changed by varying the extraction technique, in particular the degree of thermal epimerisation of catechin and

epicatechin. They selected microwave assisted extraction as the method of choice for delivering the highest extraction yields for the quantification of catechins within the shortest time, and with no epimerisation problems. Whilst using two detection systems, UV and MS, they found higher signal to noise for UV than MS, which they commented was in contradiction to their previous findings in wine (Stecher et al., 2001) where the MS gave higher sensitivity than the UV. Several possible reasons for this are that the instrumentation used may have been contaminated or not performing well, or that the different extraction technique could be extracting components that co-elute and affect the ionisation efficiency of the target metabolites in the MS chromatogram. Upon investigating samples of different origin with the LC- MS method they found differences but made no interpretation of these.

Recently, Guillarme et al. (2010a) used both UHPLC-MS QqQ and UV detection for quantification of the 8 predominant polyphenols in black tea extracts. After intensive optimisation of UHPLC parameters such as column, flow rate and gradient, the eight polyphenols in a standard mixture could be resolved within 30 seconds, with a total run time of 2 minutes per sample. However, when the method was applied to tea samples the complex matrix affected chromatographic results and the authors had to resort to a slower method with a longer chromatography column. They concluded that “when dealing with complex matrices such as tea extracts, however, which could possess hundreds of constituents, the resolving power becomes more important than throughput”. The authors compared the QqQ and UV measurements and concluded that while the ‘gold standard method for routine analysis’ (UV) was robust, the QqQ method of detection gave benefits of both sensitivity and selectivity over UV detection.

Recently, Chen et al. (2011) developed a multi-residue method for rapid determination of pesticide residues in tea by UHPLC-MS QqQ. They utilised a modified QuEChERS extraction and a long 150 mm UHPLC column to give excellent chromatographic resolution for the 65 pesticides monitored. The method had a relatively short analysis time of 18 minutes, excellent sensitivity, and good linearity across a wide dynamic range. While the authors could have chosen a shorter and faster UHPLC column given the highly complex matrix being analysed, they considered the greater chromatographic resolution available the better, thus the choice of a longer UHPLC column aided in minimising ion suppression in the ESI source from co-eluting non-target compounds.

Continued improvements in quantitative LC-MS instrumentation with respect to speed, sensitivity, and dynamic range, combined with the reduced price of these instruments have seen this technique become widespread and common place, and the development of UHPLC technology has strengthened the robustness and applicability of LC-MS analysis for quantification.

Non-targeted analyses and techniques

GC-MS:

GC-MS is a powerful technique for profiling sample composition and generating data on unknown and known volatile components. The mass spectra collected can be used to search

against commercial libraries to assist in identifying unknown components. In this section we review recent or significant examples of the application of non-targeted GC-MS to tea.

A common application of non-targeted GC-MS has been to determine if it is possible to predict the quality of tea samples from chromatographic profiles. Ponguswan et al. (2007) utilised solvent extraction, chemical derivatisation and GC-MS to profile green tea samples and predict quality rankings via their profiles with multivariate statistics. They were able to develop a model for predicting rankings from the GC-MS profiles, with higher ranking samples containing elevated levels of amino acids, quinic acid, phosphoric acid, ribose and arabinopyranose, while the lower ranked samples had higher levels of sugars such as fructose and glucose. While this methodology aided in predicting green tea quality rankings on a small number of generally primary metabolites, the authors did not mention the components associated with the health benefits of tea consumption such as the phenolics.

These authors then developed a pyrolysis GC-MS method to again predict quality rankings via their profiles and multivariate statistics in the same green tea sample set as above (Pongsawan et al., 2008). They were again able to develop a model for predicting rankings from the pyrolysis products measured by GC-MS, which had a lower level of prediction error and higher predictivity than the SDE GC-MS method described above. While the data generated from this pyrolysis technique is useful in building predictive models for quality rankings or grading applications, it does not provide the same amount of qualitative information about chemical profiles than the less destructive methods as most of the components measured are breakdown products from multiple precursors.

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Wang and Ruan (2009) investigated chemical components in green tea and their relationship with perceived quality. They combined the data from several different analytical methods and utilised statistical analyses such as Pearson's linear correlations and principal component analysis (PCA) to investigate the component/quality relationship. Free amino acid concentrations were measured by HPLC. Volatiles were extracted and quantified by steam distillation extraction (SDE) and GC-MS and chlorophylls and carotenoids were measured by UV spectrophotometry. The volatile composition and quantities varied widely. However, the combined results showed the three main contributing components to the PCA were total free amino acids, pentanal and EGCG and the model was able to predict the quality score with 77% probability. The relationship of sweetness to the amino acid levels, especially theanine (Ekborg-Ott et al., 1997), and the contribution of the catechins to astringency (McDowell and Owour, 1992) have been reported previously. These authors then investigated the chemical components in oolong tea and their relationship with perceived quality via the same methods (Wang et al., 2010). Again the volatile composition and quantities varied widely, however, the combined results showed the three main contributing components to the PCA were glutamic acid, total catechins, and benzeneacetaldehyde and the model was able to predict the quality score with 78.5% probability. The results showed that oolong tea could be partially classified by cluster analysis based on PCA. They measured many components known to correlate with quality attributes such as theanine, however the levels did not correlate with quality as observed in a previous study (Ekborg-Ott et al., 1997). While they had some success in predicting the quality of the samples with combined analyses, there are many major components still being excluded from the dataset, such as sugars and procyanidins.

Several non-targeted studies have investigated the use of GC-FID and GC-MS in differentiating tea types by profiling the volatile composition (Kato and Shibamoto, 2001; Kumazawa and Masuda, 2002; Pripdeevech and Machan, 2011; Wang et al., 2008).

An early example by Kato and Shibamoto (2001) investigated the major volatile constituents in green teas from various Southeast Asian countries. They used GC-FID to measure levels of volatiles extracted from tea by SDE and GC-MS to identify the major components. About 100 components were detected in the samples, and although they chose to quantify only 11 of these, they were able to detect a relationship between increased tea quality and increased levels of linalool and hexanal, and determine that the teas from Laos and Myanmar contained heterocyclic compounds, such as pyrazines, which were formed during high temperature processing. The detection of the pyrazines in these samples is an excellent early example of the potential of non-targeted analysis and its application to sample differentiation.

Wang et al. (2008) used a combination of solid-phase microextraction (SPME) and GC-FID to discriminate teas with different degrees of fermentation, by profiling volatiles and performing GC-MS for component identification and GC-odour port sniffing to characterise the components flavour. Higher levels of volatiles were measured in the fermented compared to unfermented tea. They identified approximately 70 components and their flavour attributes. Only a small number showed significant differences in composition between the unfermented and semi/fully-fermented teas. However, they were able to differentiate the teas by combining several volatile components, particularly trans-2-hexenal, benzaldehyde, methyl-5-hepten-2-one, methyl

salicylate and indole. They also monitored the catechin content via LC-UV but could not differentiate green and oolong teas via this measurement in their sample set.

More recently, Pripdeevech (2011) used GC-MS to fingerprint the volatile composition of non-fermented and semi-fermented teas from Thailand, identifying between 50-70 volatile components in the different oils extracted by SDE. They observed higher levels of volatiles in the fermented teas compared to non-fermented teas, in particular much higher levels of cis-jasmone, trans-nerolidol and indole in the fermented, and decreased levels of hotrienol, a compound associated with the green fresh aroma of tea.

While these techniques have been successful in differentiating between tea type, generally only a limited number of components have been selected, with very little crossover of components between the studies. This suggests that there is significant variation in the volatile profiles of tea, and the studies reported above were detecting the variety within the experimental sample sets used rather than consistent volatile biomarkers for fermentation and tea type. Because GC-MS will not detect most of the major secondary metabolites such as flavanoid glycosides due to their low volatility, the methodology is highly biased towards lower molecular weight volatile components.

LC-MS:

Many studies have reported the detection and identification of components in tea beyond the list of standard metabolites using LC-MS. More recent studies have begun to develop methods for differentiating and profiling tea types, detecting many more metabolites than just the phenolic

components. Potential applications of these methodologies are to grade tea quality or determine the geographical origin of tea samples. In this section we review recent or significant examples of the application of non-targeted LC-MS to tea.

Several studies have investigated the potential of using LC-MS to profile tea metabolites and relate the measurements to e.g. the composition of different tea types or processing methods.

Xie et al. (2009) utilised a UHPLC-qToFMS approach for a rapid (10 minute) reversed phase analysis of extracts to evaluate the quality of green, black and pu-erh tea. They were able to differentiate the three tea types by multivariate statistics utilising all peaks observed in the chromatogram, and could resolve the teas on the basis of simple spectroscopic measurements of pigments such as theaflavins, thearubigins and theabrownin. Following on from this, they investigated the effects of consumption of pu-erh tea on urinary profiles, detecting a perturbation in the urinary profile upon ingestion, and significant delays in the profile returning to baseline (over a two week period), suggesting an ongoing alteration of the resident gut microflora. The differentiation of tea samples by all peaks in the MS chromatogram (which include pigment peaks) was to be expected as samples could be differentiated by spectrophotometric analysis alone. However, the analysis while extensive was not comprehensive as only components eluting by reversed phase chromatography and detectable in +ve ESI mode were monitored.

Ku et al. (2010) used a metabolomic approach with analysis by LC-ITMS to monitor changes in the effects of manufacturing type and number of post-manufacturing fermentation years on pu-erh tea composition. The reversed phase chromatographic run time was 40 minutes and the MS was operated in –ve ESI mode only; 377 peaks were detected, and 343 of them were affected by

the ripening process used in pu-erh tea manufacturing. They found significant negative correlations between post-fermentation year and levels of polyphenolic compounds such as EGCG, EGC and ECG. By using an ITMS instrument, they were however limited by the scan speed cycle of a trap filling instrument, thus limiting chromatography speed. Again, the authors potentially missed significant components by only operating the MS system in one polarity mode, and with one chromatographic phase.

Tea quality has traditionally been assessed by skilled specialists who evaluate the products quality on several attributes, such as the appearance of the leaves and the taste and aroma of the brew. Recently, there has been considerable interest in using analytical methods to correlate instrumental measurements with taste panel tea quality assessments, with LC-MS techniques being investigated to predict the quality of tea samples. Pongsuwan et al. (2008a) used UHPLC-ToFMS to develop a high-throughput technique for the comprehensive analysis of Japanese green tea and to relate the results of metabolite profiling to the quality ratings of tea samples. Samples were run on a reversed phase UHPLC column with a 10 minute runtime in both –ve and +ve ESI mode, similar to the methodology used by Xie et al. (2009) above. They excluded processing the +ve ESI data as they found poor stability of ionisation, whereas Xie et al only operated in +ve ESI data and made no mention of ionisation stability issues. Many co-eluting compounds were observed but after chromatographic peak detection with peak picking software they extracted 1560 components from the chromatogram. They found that both high and low grade tea gave the same chromatographic peaks; however there were differences in intensities of the components. Most components were reported as “unknown” although they were able to

generate a model to predict tea quality based on the metabolite profile, with significant biomarkers of Japanese green tea quality being EGC, EGCG and ECG. However, their assessment of quality factors was incomplete as some components important to quality such as caffeine (Luypaert et al., 2003) can be detected only in +ve ESI MS mode.

One of the problems in the LC-MS analyses of crude plant extracts is the complexity of the extracts (Sumner et al., 2003). Without further purification steps such as solid phase extraction, the extract will contain a wide range of both highly polar and non-polar compounds, such as salts, mono- and disaccharides, small organic acids and long chain fatty acids (Dettmer et al., 2007). There is generally a wide concentration range between primary and secondary metabolites in such crude extracts, so when a sensitive method is required it is unavoidable that high levels of some components will be injected into the chromatographic system. These highly complex extracts can potentially quickly contaminate the ion source interface and cause decreasing signal intensity. A key strategy employed to avoid rapid contamination of the mass spectrometer in reversed phase chromatography is to divert the solvent front (where the highly polar components will elute) and the column clean-up stage (where the non-polar components will elute) of the chromatogram away to waste. A potential alternative strategy to simplify the complexity of crude extracts is by 2-D LC.

Kalili and de Villiers (2010) investigated the separation of green tea phenolics by comprehensive 2-D LC (LC × LC). An offline system was designed; based on hydrophilic interaction liquid chromatography (HILIC) as the first dimension, and reversed phase (RP) C18 as the second dimension. The phenolics were detected online with PDA, fluorescence or in -ve ESI with a q-ToFMS. HILIC chromatography is based on differences in polarity with weakly polar

compounds eluting before highly polar compounds. Thus gallocatechins were retained longer than catechins, and the procyanidins eluted in order of molecular weight. They observed that compounds which exhibited low retention on the HILIC column were generally spread across the entire RP-LC chromatogram. These two chromatographic systems are not compatible together online as the primary eluent in either mode is a strong elution solvent in the secondary mode, thus the HILIC was carried out separately and small volumes of fractions collected from the HILIC column were injected onto the RP column. Due to the effective clean-up of the procyanidin fractions by offline HILIC chromatography, they were able to identify many components in the green tea samples including the first reported detection of proanthocyanidin pentamers, hexamers and heptamers. The authors claimed a high practical peak capacity due to the low degree of correlation between the two chromatographic mechanisms, thus a higher theoretical number of components that can be resolved by combining the two columns (HILIC and RP-LC separation), which can offer improvements in resolving power for complex samples compared to 1-D LC.

Direct infusion MS:

Direct infusion MS (i.e. no prior chromatographic separation of the sample) gained popularity in recent years in the areas of plant and microbiological metabolomics (Beckmann et al., 2008; Koulman et al., 2007). Surface desorption is another direct MS approach where the surface of a solid sample is subjected to ionisation methods near the entrance to the MS.

One of the more innovative methods attempted for differentiating teas was performed by Chen et al (2007) using surface desorption APCI-MS. The method involved placing a leaf in front of the

MS entrance capillary and using a moist charged gas stream containing H₃O⁺ ions to ionise compounds off the surface of the leaf and into an ITMS. They were able to differentiate between green, oolong, and jasmine teas by PCA on the raw mass spectral data. However, although the technique was fast and effective in fingerprinting the tea samples, the spectra generated were heavily biased towards components that were on or very near the surface layer of the leaf and appeared biased towards low molecular weight components. This technique suffers the pitfalls of no chromatographic resolution of the components. With the fingerprint spectrum provided being only of nominal mass, components with the same nominal mass but different molecular formula and exact masses (such as theanine (C₇H₁₄N₂O₃) and arginine (C₆H₁₄N₄O₂)) cannot be differentiated and will contribute to the same nominal mass bin. This approach is not quantitative as the surface components of the leaf will vary across the leaf and across different samples.

MALDI MS:

Menet et al. (2004) used MALDI-TOF to analyse black tea extracts for theaflavins and thearubigins, specifically using delay pulsed ion extraction in the ion source to gain some fragmentation and infer likely structural characteristics for these compounds. While MALDI has the advantages of speed, ease of use, and adequate sensitivity, it again suffers from no chromatographic resolution, relying solely on mass resolution with the ToF. The ionisation of compounds is highly dependent on the matrix used, so spectra can be heavily biased on a particular class of compounds due to the matrix selected.

CONCLUSIONS

There have been substantial improvements in the reliability, sensitivity, and speed of MS based analytical instrumentation over the past 10 years. The cost of these instruments has reduced and this combined with the improved usability due to better computing software and hardware means more analysts have access to better quality and more advanced instrumentation and data.

The publications covered in this review of tea literature utilise many types of mass spectrometers for both targeted and non-targeted analyses. The main application of MS systems for targeted analyses in tea is to measure the phenolic components using LC-MS, as these are considered important bioactive components due to their known antioxidant activity. LC-MS offers a fast, selective, and sensitive detection mechanism for the phenolics as they chromatograph and ionise well with traditional LC-MS solvents and conventional reversed phase columns. The range of volatile components able to be resolved and detected by GC-MS is increasing as 2-D GC methodology improves and MS detection techniques become more sensitive; e.g. the coupling of GC-QqQ. Key areas of research for non-targeted analyses by both GC- and LC-MS are sample differentiation, such as determining key components for differentiating tea type, and predicting tea quality rankings by generating prediction models that compare as many components as possible against quality rankings provided by tea tasters. This area of non-targeted analyses is becoming more widespread as chromatography/MS instruments can resolve more complex mixtures faster, and data processing software improves to handle the vast amount of data that are generated.

In recent years, many chromatographic methods have been developed for analysing primary and secondary metabolites in tea. LC methods are by far the most common techniques used, due to their versatility, high separation power, and capability to handle many different and sensitive detectors. Technological improvements in areas such as chromatography have also provided significant benefits resulting in increased sensitivity and reduced analysis times. One such example is the development of UHPLC columns which provide significant advantages over conventional LC for both targeted and non-targeted analysis. For any stationary phase type, a comparable UHPLC column offers a greater number of theoretical plates, higher peak capacity, and the highest separation quality available by LC. In the area of metabolite profiling by LC-MS, UHPLC results in datasets with higher number of components detected and better quality and more reliable data. UHPLC methods are generally significantly faster than conventional methods, usually 3-5 times faster but in some cases up to 15 times faster, and retention times are usually more stable. The narrow peaks give increased sensitivity and are easily handled by fast scanning MS instruments such as a ToF, but can be detrimental to the quality of the quantitative data that can be extracted from slow scanning MS instruments, like an ion-trap, due to a lower number of points across the chromatographic peak. The decrease in run time per sample offers an opportunity for analysts in a busy laboratory to run samples more than once, perhaps examining the samples in both +ve and -ve mode or utilising another ionisation source or chromatographic column/system, thus increasing the number of components detected per sample. This is an important point when considering that most of the non-targeted literature reviewed generally only analyse samples in one ionisation mode and chromatographic phase, limiting (and biasing) the number of metabolites that were detected. Given that some of the goals of non-

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targeted analyses are defining composition, product quality factors, or even understanding which component or class of compounds are responsible for particular health benefits that are associated with tea products, then a comprehensive analysis is important as key factors can be omitted by the choice of analytical method.

Another area of future technical advance is likely to be in the improved coupling of chromatographic techniques, such as 2-D GC or LC. This again gives significant advantages in being able to properly resolve and detect a greater percentage of the multitude of components in the highly complex tea matrix. Finally, as instrumentation continues to improve, the savvy analyst will ensure they gain the highest quality results achievable by using the latest, highest quality and most cost effective chromatographic and mass spectrometric technologies available, tools which will be invaluable in the complex area of tea primary and secondary metabolite research.

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Table 1: Summary of common commercially available sub 2 μm particle size UHPLC phases available rated for >11,000 psi

Vendor	Available phases	Particle size
Agilent	C18, C8, CN, phenyl	1.8 μm
ES Industries	C18, PFP, HILIC, diol, silica	1.8 μm

Grace-Davison	C18, HILIC, silica	1.5 µm
Interchim	C18, HILIC	1.7 µm
Knaeur	C18, C18-aq, C8, PFP, biphenyl, CN, silica	1.9 µm
Macherey-Nagel	C18, C8	1.8 µm
Restek	C18, C8, PFP, biphenyl, CN, silica	1.9 µm
Thermo	C18, C8, C4, PFP, CN, phenyl, amino, WAX ^a , SAX ^b , silica	1.9 µm
Waters	C18, C8, phenyl, HILIC, amide	1.7 and 1.8 µm

^a Weak anion exchange

^b Strong anion exchange

Table 2: Instrumental techniques used in analysing tea metabolites

<i>Instrumental technique</i>	<i>Example of application</i>	<i>Reference</i>
Targeted		
GC-MS	Pesticide screening of green tea	(Ochiai et al., 2005)
LC-ITMS	Identification and comparison of phenolics in oolong tea	(Dou et al., 2007)
LC-MS	Analysis of flavonoids in tea of	(Sultana et al., 2008)

	different origins	
LC-QqQMS	Analysis of mycotoxins in tea products	(Monbaliu et al., 2010)
LC-QqQMS	Rapid analysis of catechins	(Guillarme et al., 2010a)
Untargeted		
GC-MS	Predict rankings of Japanese green tea	(Pongsawan et al., 2007)
LC-ITMS	Analysis of phenolics in green and black tea	(Del Rio et al., 2004)
LC-ITMS	Characterising galloylquinic acids of green tea	(Clifford et al., 2007)
DAPCI-MS	Differentiation of tea by surface desorption	(Chen et al., 2007)
MALDI-ToF	Analysis of theaflavins and thearubigins from black tea	(Menet et al., 2004)
LC-qToFMS	Characterization of Pu-erh tea using chemical profiling	(Xie et al., 2009)

Captions for Figures:

Figure 1: Common metabolites reported in tea

Figure 2: Principle differences between green, oolong and black tea processing

Figure 3: Comparison of the different detection techniques used to monitor the HPLC separation of a crude green tea extract. The peak on the m/z 459 is gallocatechin 3-*O*-gallate, and its MS2 and MS3 spectra are shown.

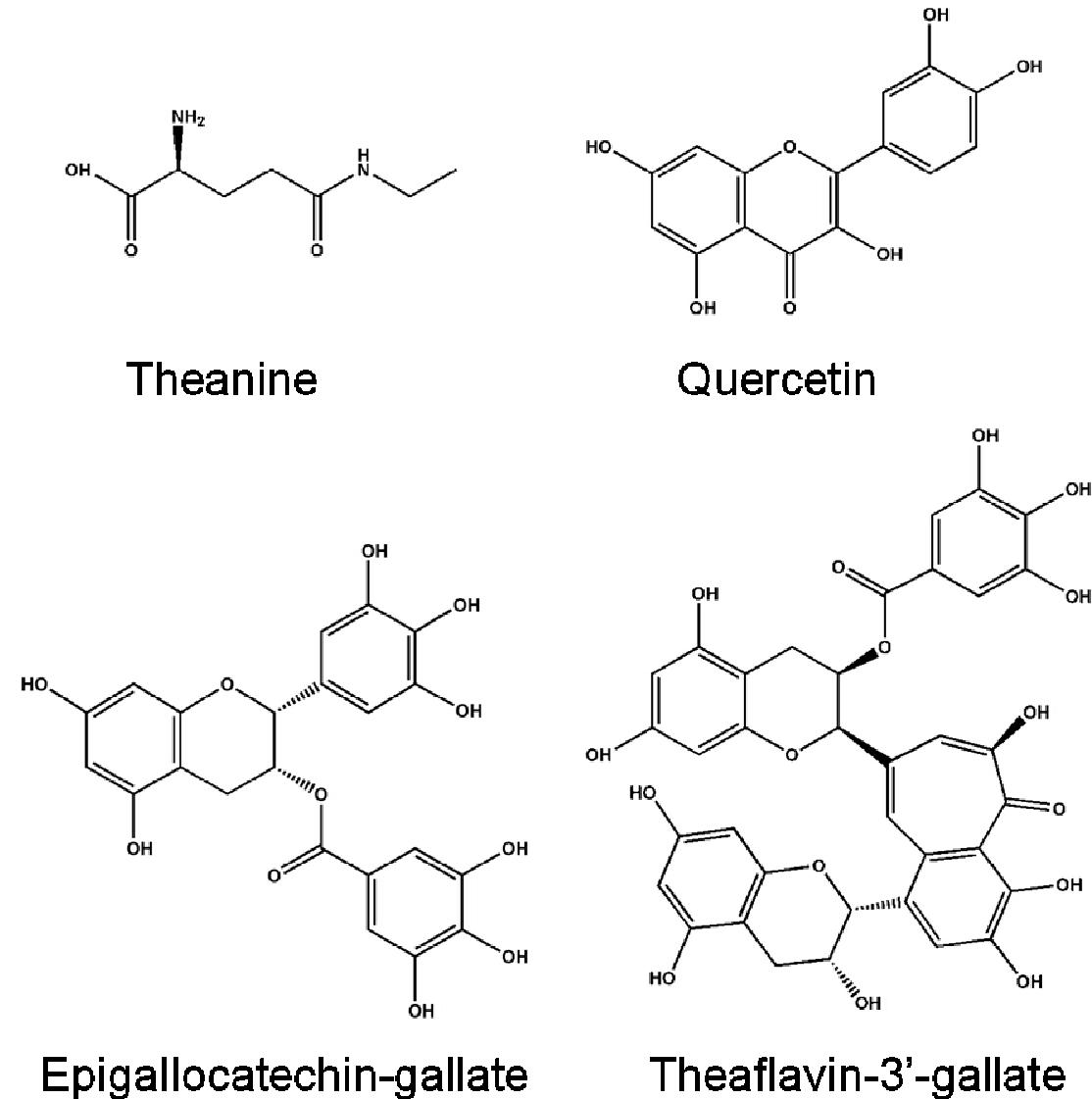


Figure 1

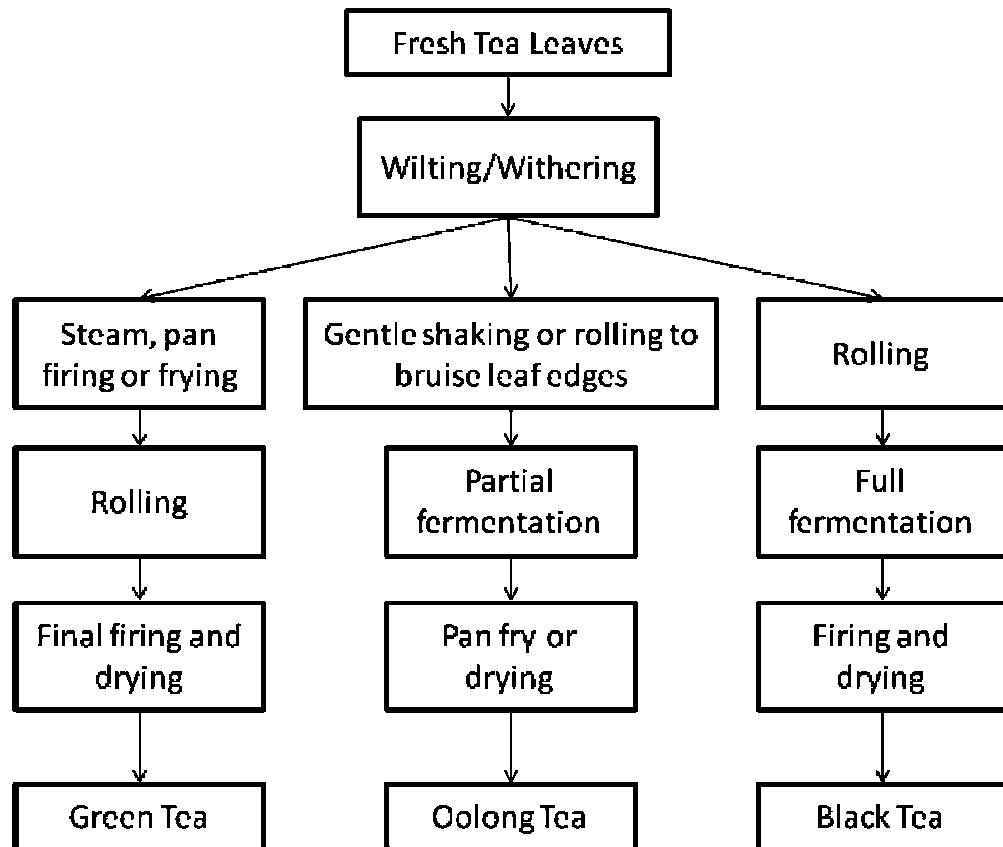


Figure 2:

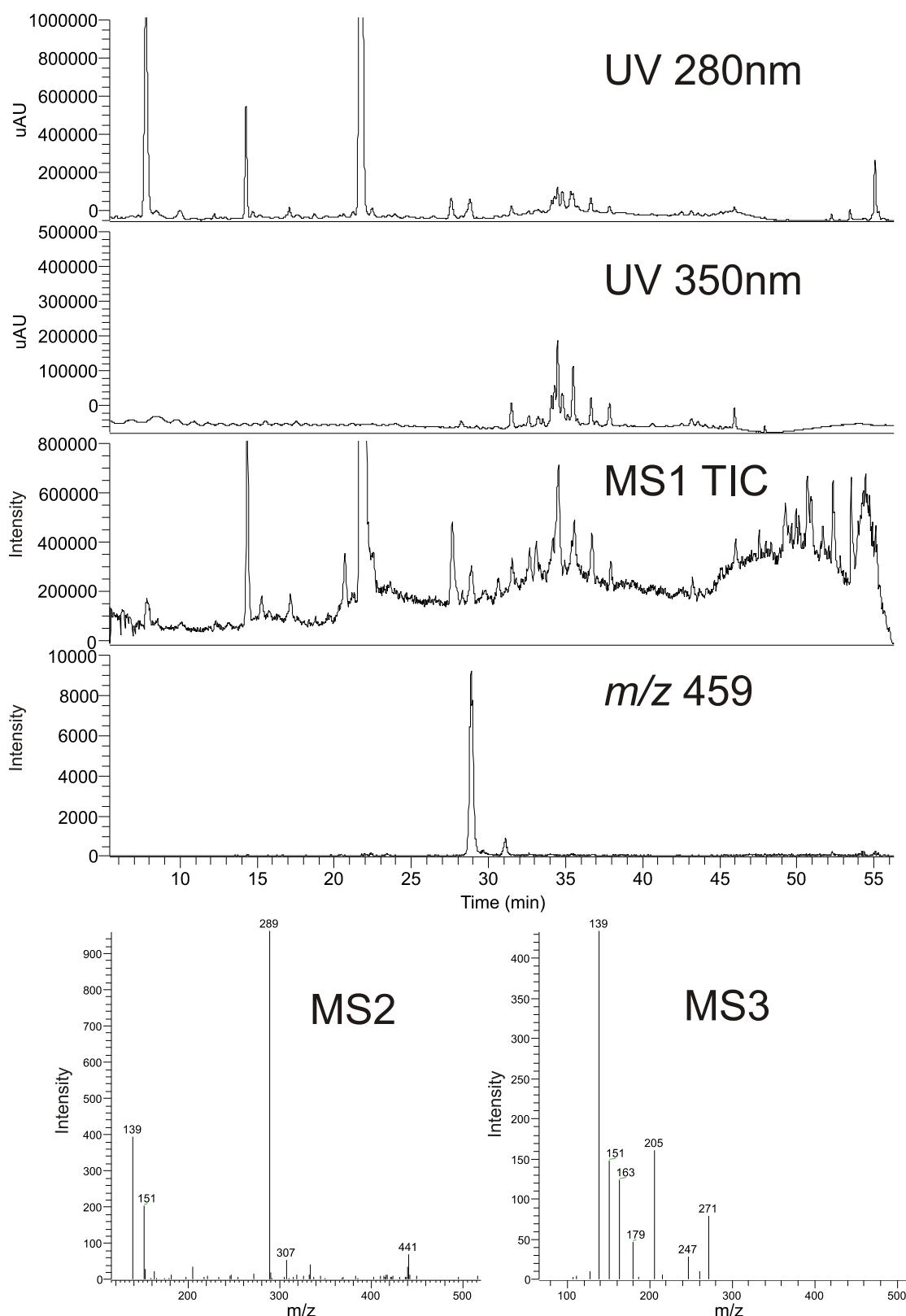


Figure 3