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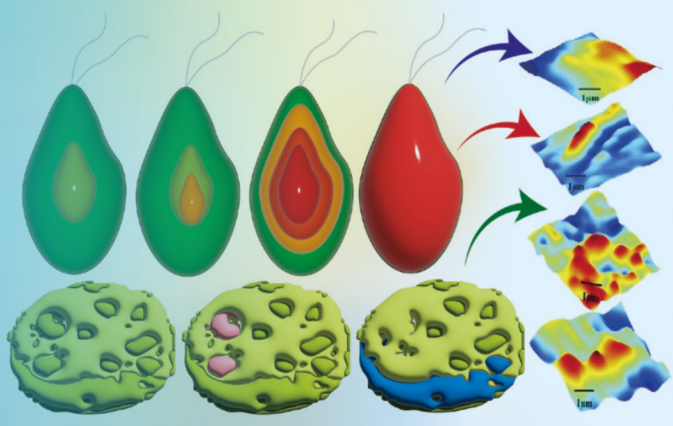

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Editors

Plant Chemical Compositions and Bioactivities

Humana Press

Methods and Protocols in Food Science

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Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science.

Volumes and chapters will be organized by field and presented in such way that the readers will be able to reproduce the experiments in a step-by-step style. Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol will be clarified.

Plant Chemical Compositions and Bioactivities

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ISSN 2662-950X ISSN 2662-9518 (electronic)
Methods and Protocols in Food Science
ISBN 978-1-0716-3937-5 ISBN 978-1-0716-3938-2 (eBook)
https://doi.org/10.1007/978-1-0716-3938-2

Preface to the Series

Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science. The series is unique as it includes protocols developed, validated, and used by food and related scientists, as well as theoretical basis are provided for each protocol. Aspects related to improvements in the protocols, adaptations, and further developments in the protocols may also be approached.

Methods and Protocols in Food Science series aims to bring the most recent developments in research protocols in the field as well as very well established methods. As such the series targets undergraduate, graduate, and researchers in the field of food science and correlated areas. The protocols documented in the series will be highly useful for scientific inquiries in the field of food sciences, presented in such way that the readers will be able to reproduce the experiments in a step-by-step style.

Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol is clarified. Then, an in-depth list of materials and reagents required for employing the protocol is presented, followed by a comprehensive and step-by-step procedures on how to perform that experiment. The next section brings the do's and don'ts when carrying out the protocol, followed by the main pitfalls faced and how to troubleshoot them. Finally, template results will be presented and their meaning/conclusions addressed.

The *Methods and Protocols in Food Science* series will fill an important gap, addressing a common complain of food scientists, regarding the difficulties in repeating experiments detailed in scientific papers. With this, the series has a potential to become a reference material in food science laboratories of research centers and universities throughout the world.

Campinas, Brazil ***Anderson S. Sant'Ana***

Preface

The international community's attention has lately focused on the emerging technologies for fast and accurate analysis of food products and their quality, which are considered to be ecofriendly and have significant reproductivity impacts. The potential chemical-free and accurate methods that can track the accumulation of these compounds during the industrial process could open the way through the smart systems for producing high-quality and precise final products. Furthermore, scientists are trying to find instant and non-invasive techniques to explore the scientific principles of phytochemicals' functional principles, especially bioactive macromolecules and micromolecules. As an important example, the quality of tea product during its fermentation process is based on the accumulation of some compounds like theaflavins (TF), thearubicin (TR), and theabrownin (TB), which have significant impacts on its food industrial systems. In addition, many studies have reported that these kinds of phytochemicals have high sensitivity to the different analytical protocols because of their isomers' activity and their unique chemical structure. Besides, the plant and animal health-related key components like carbohydrates, lipids, and proteins are precursors of essential pathways and are novel sources of bioactive molecules that are affected by oxidation and other physical and chemical processes. These physicochemical changes could affect the quality of the final food product. Therefore, tracking these mechanisms could positively affect the field of the food chemistry and its industrial scale research, especially in the functional prebiotic carbohydrate, protein, and lipid production. As an example, microalgae lipids provide several aspects in the food science and nutrition area as a viable alternative source in food technology. They could have up to 40% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) of its fatty acids as functional bioactive molecules. And the real-time study of their combination with the other natural safe molecules (e.g., polyphenols and flavonoids) could raise their bioactivities, while caring about their storage, processing, and quality efficiency by using the novel analytical technologies.

The vision for this handbook is to provide food researchers the current state of advanced destructive and non-destructive techniques for measuring the chemical composition, functional bioactivities, and physicochemical changes and explaining the functional mechanisms for facilitating the tracking of the functional molecules during the different industrial processes. Besides, the development as well as providing practical methodologies that demonstrate the integration of the analytical methods and protocols are discussed in details. The chapters deal with a critical discussion of both laboratory and food industrial topics, with each chapter containing both a discursive section along with a detailed methods section. The handbook's topics include sample preparation, emerging extraction technologies protocols, analytical methodologies for phytochemicals profiling; multi-way models in food data analysis; and the integration of omics for biochemical analyses understanding.

Along with the various chemical-free and chemical-dependent methods covered in the individual chapters, the handbook also includes an extensive bibliography that details current existing literature in the fields like:

- Food materials science
- Natural bioactive products physicochemical analyses
- Nano- and microsensors and sensation
- Green chemistry
- Analytical methods
- Electrochemical sensors

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Chapter 1

Application of FT-IR, and Hyperspectral Analytical Protocols for Tea Leaves Natural Catechins and Caffeine Detection and Evaluation

Zhenxiong Huang, Xiaoli Li, and Mostafa Gouda

Abstract

The contents and distribution of catechins and caffeine, strongly reflect the physiology and nutrition of tea. In recent years, spectroscopy technology has been widely applied to the detection of tea content. Based on spectroscopy technology (Fourier transform infrared (FT-IR) spectroscopy and hyperspectral imaging technique) and chemometrics, rapid and nondestructive determination model of catechins and caffeine content can be developed. In this chapter, we present a protocol for determining catechin and caffeine content in tea leaves by spectroscopy technology coupled with chemometrics. This analytical method could provide quick and efficient detection of catechin and caffeine in fresh tea leaves, which have great impacts on the cultivation of tea trees and the selection of raw materials for tea processing.

Key words Tea leaves, Caffeine, Catechin, FT-IR spectroscopy, Hyperspectral imaging, HPLC

1 Introduction

Tea has a long history in the world and being used in about 160 countries every day for drinking [1]. By reason of the pleasant flavor and various health promoting functions contributed, tea has become one of the most consumed nonalcoholic beverages [2]. Catechins and caffeine are two significant bioactive chemicals in tea leaves, and also are the basis of high-quality tea leaves. Catechins have been shown to have a great impact on the color, fragrance, oral astringency, and bitterness of tea. Moreover, catechins can inhibit bacteria and resist insects by reacting with protein. Caffeine, which can stimulate the central nervous system and enhance the excitability of the cerebral cortex, is one of the most important biological ingredients of tea [3]. Therefore, it is necessary to determine the catechins and caffeine contents of leaves because it can offer suggestions for optimizing high-quality tea processing by improving the raw material.

Mostafa Gouda et al. (eds.), *Plant Chemical Compositions and Bioactivities*, Methods and Protocols in Food Science,
https://doi.org/10.1007/978-1-0716-3938-2_1,

The traditionally detected in contents of tea catechins are by chromatography and capillary electrophoresis methods [4, 5], and caffeine are detected by the iodometric method and ultraviolet spectrophotometry [6, 7]. Among them, high-performance liquid chromatography (HPLC) is a widely accepted method. However, these methods are time-consuming, complicated, and reagent-consuming in large-sample detection [8]. In recent years, spectroscopy technology has proven to be a powerful analytical tool in the determination of tea components. Hyperspectral imaging technique is an emerging technology that integrates both digital image and spectroscopy technology in one system to acquire spatial and spectral information of tea leaves [9]. A spectrum for each pixel and an image for each narrow band can be acquired which enable this system to reflect componential and constructional characteristics of tea leaves and their spatial distributions [10]. FT-IR spectroscopy is used to obtain high spectral resolution data of absorption over a wide infrared (IR) spectral range where the specific frequency of molecular absorption that matches the transition energy of the vibrational frequency of a bond or group in catechins and caffeine of tea leaves [11]. The peaks at around 2852 and 2924 cm^{-1} could be assigned to C–H bonds of CH_3 groups, which are related to caffeine [12]. The main absorption peaks of samples in the fingerprint region were at 825, 1036, 1145, 1317, and 1519 cm^{-1} consistent with the spectral peaks of catechins [13]. The absorption peak at 1369 cm^{-1} consistent with the spectral peaks of caffeine [14]. Based on FT-IR spectroscopy and hyperspectral imaging technique, quantitative detection and distribution visualization of catechins and caffeine in tea leaves can be achieved.

Here, we explain the potential of FT-IR spectroscopy for determination of catechins and caffeine with no organic reagent extraction. Meanwhile, hyperspectral imaging technique was employed to determinate the catechins and caffeine content and map the spatial distribution of catechins and caffeine in tea leaves.

2 Materials

The chemicals and solvents used must be of HPLC analytical grade. Unless otherwise specified, all processes and solutions can be carried out at room temperature.

2.1 Chemicals

1. Acetic acid, from Thermo-Fisher (Waltham, MA, US).
2. Acetonitrile, from Thermo-Fisher (Waltham, MA, US).
3. High-purity water, from Watsons (Hongkong, China).
4. Potassium bromide, from Labio (Shanghai, China).

2.2 Mobile Phases

1. Mobile phase A was acetic acid, acetonitrile, and high-purity water in proportion of 0.5:3:96.5 (v/v/v).
2. Mobile phase B was acetic acid, acetonitrile, and high-purity water in proportion of 0.5:30:69.5 (v/v/v) (*see* **Notes 1–3**).

2.3 HPLC Materials

An HPLC analysis system (LC-20AD, Shimadzu, Kyoto, Japan) included an SIL-20A automatic injection valve and an SPD-20A UV-visible detector. The extracted solution of samples were injected into a Shim-packed column (e.g., 5 μm, 4.6 mm × 250 mm, or similar) (*see* **Note 4**).

2.4 FT-IR Spectrometer

An FT-IR spectrometer (Nicolet Is10, Thermo Fisher Scientific, Waltham, Mass.) equipped with a TGS detector. The scanning range was 7800–349 cm^{-1} (*see* **Note 5**).

2.5 Hyperspectral Image System

Hyperspectral image system included ImSpectorV10E (Specim, Finland) (*see* **Note 6**), a 12-bit CCD camera (Hamamatsu, Japan), two 150W tungsten halogen lamps (Fiber-Lite DC950 Illuminator, Dolan Jenner Industries Inc., USA), and a conveyor system (*see* **Note 7**). The scanning range was 380–1030 nm. Noise of spectra are usually removed during the analysis process.

3 Methods

3.1 Extracting of Samples for HPLC

1. Tea leaves are freeze-dried (FreeZone, Labconco, Kansas City, Mo.) and milled into a powder with a grinder (FW100, TY Instrument Co. Ltd., Shanghai, China).
2. The ground of tea leaves were sieved through a 40-mesh sifter.
3. 0.1 g of sieved tea powder and 25 mL of pure water was added into test tube and stirred.
4. The tube was placed in an 85 °C water bath for 20 min.
5. The supernatant was filtered with a 0.22 μm microporous hydrophilic membrane.

3.2 Operating Procedures for HPLC

1. Inject the filtered and degassed mobile phase into the storage tank.
2. Rinse the metal filter with the mobile phase, and then immerse the filter in the mobile phase of the storage tank.
3. Place the liquid storage tank at a certain height to avoid unnecessary falls caused by handling and other operator movements.
4. Start the HPLC in the following order: pump → detector → HPLC software → set the software parameters according to the preplanned method, such as analysis time, detection wavelength, flow rate, etc.

5. Start the pump and run for 5 min, mainly to eliminate bubbles in the system. After completion, close all exhaust valves.
6. Running the mobile phase at a fixed rate according to the predetermined speed, and follow the baseline until it stabilizes. Then, monitoring can be carried out on computer software.
7. After the baseline is stable, set the running parameters of tea sample in the software, such as flow rate and analysis time. The analysis time may vary due to factors such as sample, flow rate, and column length.
8. After setting the parameters, inject a fixed volume of sample solution into the injection valve, and then start the injection command in the software. The sample solution in the injector enters the chromatographic column with the mobile phase.
9. Monitor various readings through software, and when the detector detects all peak values of the sample, stop running and set a new injection. Before analyzing the next sample, it is recommended to allow 5–10 min for the mobile phase to pass through the chromatographic column in order to clean the residue of the previous sample. After confirming baseline stability, proceed to the next injection.
10. After the analysis is completed, first turn off the detector, then turn off the pump, and finally turn off the software.

3.3 HPLC Data Analysis

1. Linear standard calibration curves of catechins and caffeine were established according to the injection volume of the standards and HPLC peak areas.
2. Catechins and caffeine contents in the tea extract were initially identified by comparing the retention times with those of the standards.
3. Catechins and caffeine contents were then calculated from the corresponding peak areas and the external standard calibration curves.

3.4 Constructing Determination Models Based on FT-IR Spectra

1. Ball milling were utilized to milled freeze-dried leaves into powder (*see* **Note 8**).
2. 0.01 g of sample was uniformly mixed with 0.49 g of dried KBr powder (*see* **Note 9**). For this step, we recommend grinding under an infrared heat lamp to minimize the change in moisture content.
3. 0.1 g of evenly mixed powder was pressed at 15 MPa for 15 s using a tableting machine (*see* **Note 10**).
4. Turn on FT-IR spectrometer power supply, display, computer, and other power switches in order.

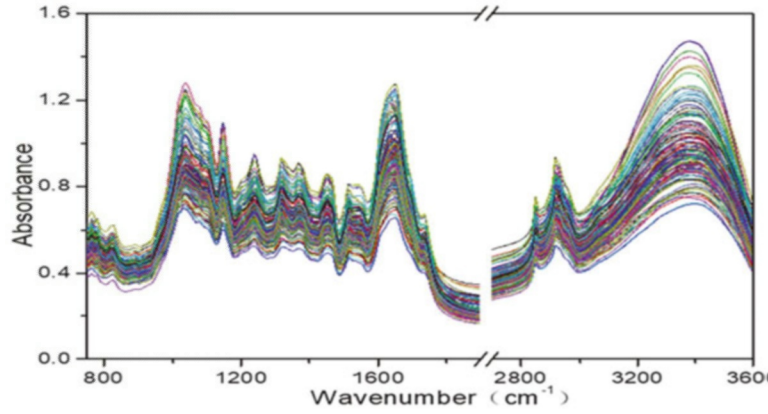

Fig. 1 FT-IR spectra of tea leaves

5. Double click on the desktop shortcut key "OMINIC" to enter the OMINIC workstation.
6. After the instrument is automatically initialized, when the "optical table status" appears in the upper right corner, the instrument can be preheated for about 10 min before measurement.
7. Click on "Experimental Settings" under the "Collection" menu. Scanning time and spectral resolution were set before collecting spectra. For this step, we recommend 32 times and 4 cm^{-1} for scanning time and spectral resolution separately. Final format: select "transmittance".
8. Setting the spectral range of 4000–400 cm^{-1} in the "Optical Table".
9. Opening the sample room cover, place the blank control on the sample rack in the sample room, and cover the sample room cover. Click "Collect Background" under the "Collect" menu to pop up a dialog box. Click "OK" to perform background scanning (*see* **Note 11**).
10. Opening the sample chamber cover, remove the blank control, place the sample prepared by the appropriate method on the sample rack in the sample chamber, and cover the sample chamber cover. Click on "Collect Samples" under the "Collect" menu bar to scan the samples. After the data collection is completed, the "Data Collection Complete" window will pop up and click "Yes". The spectra of tea leaves are shown in Fig. 1 (*see* **Note 12**).
11. Selecting "Save" under the "File" menu to open the "Save As" window. Selecting "Save Type" as "CSV Text (*. CSV)" from the drop-down menu, and entering the name of the saved file, and click "Save".

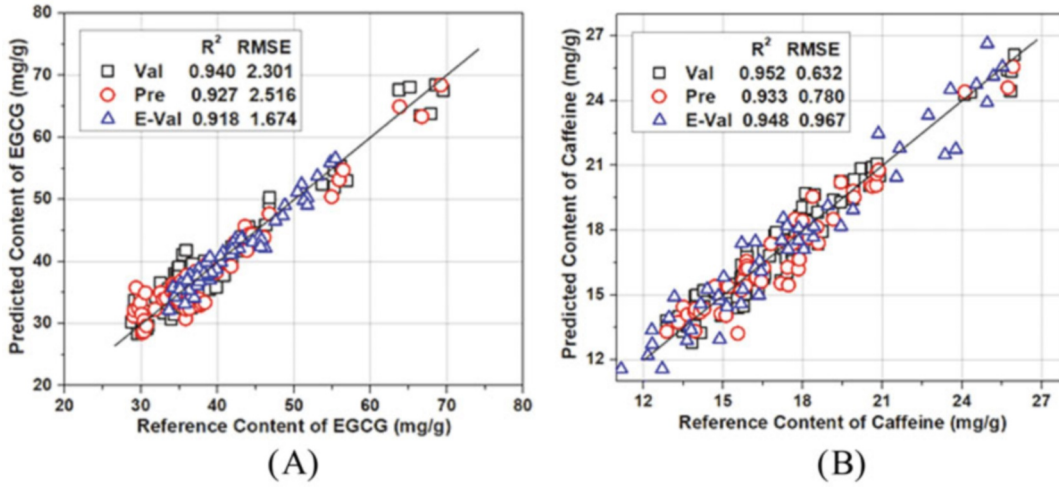

Fig. 2 Determination models of (**a**) catechins and (**b**) caffeine content

12. After collecting the spectrum, removing the sample cell, loosing the screws, putting on the fingertips, and carefully remove the salt sheet. First, wipe the liquid with soft paper, drop anhydrous ethanol, and wash off the sample. Avoid washing with water. Then, polishing with talcum powder and anhydrous ethanol under an infrared lamp. Finally, washing the surface with anhydrous ethanol, wiping it dry, drying it, wiping the mold, sample holder, etc. as required, and storing the two salt tablets in a dryer.
13. Choose File>Exit to exit the program.
14. Selecting "Shut down" from the "Start" menu on the computer desktop and a safe shutdown prompt will appear.
15. Turning off computer power, turning off the FT-IR spectrometer power, and turning off the regulated power supply.
16. Determination models for catechins and caffeine were constructed based on FT-IR spectra data and the chemical value of HPLC (*see* **Note 13**), shown in Fig. 2.
17. Appropriate preprocessing methods can improve the performance of the model (*see* **Note 14**).
18. Feature wavenumber extraction can improve the running speed of the model while ensuring accuracy, and making the model easier to develop portable equipment (*see* **Note 15**).

3.5 Constructing Determination Models Based on Hyperspectral

1. Turning on the halogen lamp, and the power of mobile platform and spectrometer.
2. Turning on the computer and running the software of "Isuzu Optics Crop".

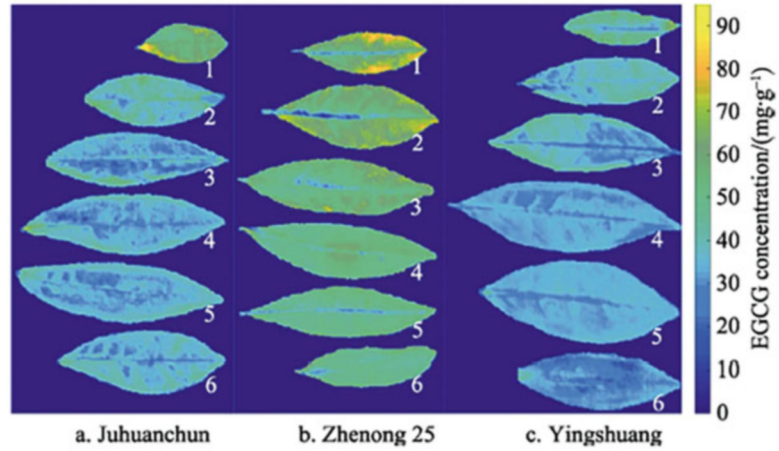

Fig. 3 EGCG concentration distribution diagram from 1st to 6th leaves of different cultivars

3. Setting the exposure time of the hyperspectral camera, the speed of the moving platform, and the distance between the lens and the sample to ensure that the collected hyperspectral images do not produce distortion.
4. The hyperspectral image of tea samples was captured through the movement of the conveyor belt.
5. White reference images (I_{white}) were obtained by collecting spectra of Teflon white board with 99% reflectance.
6. Dark reference images (I_{dark}) were obtained by closing the tungsten halogen lamps.
7. Tea leaves were placed flat on a black nylon baseplate for scanning to acquiring hyperspectral image of tea sample (R_{raw}).
8. Images of tea leaves were calibrated (R_{cal}) by the equation of $R_{cal} = (R_{raw} - I_{dark})/(I_{white} - I_{dark})$.
9. Regions of interest (ROIs) is chosen for each tea leave.
10. Mean spectra of each ROI is calculated as the representative spectra of each tea leaves.
11. Determination models for catechins and caffeine were constructed based on hyperspectral data and the chemical value of HPLC (*see* **Note 13**).
12. Inputting the spectra of each pixel into the determination model, the distribution of catechins (*see* Fig. 3) and caffeine in the entire leaves can be obtained.
13. Appropriate preprocessing methods are always welcome and can improve the performance of the model (*see* **Note 14**).
14. Feature band extraction can make the model easier to be used in portable equipment by improving the running speed of the model (*see* **Note 15**).

4 Notes

1. The volumes of mobile phases to be prepared depends on the number of extracted samples to be analyzed in each specific study.
2. The HPLC system was calibrated according to the verification regulations for liquid chromatographs (JJG 705-2014) when you are going to start using the HPLC system.
3. Elution conditions were set to change the proportion of mobile phase B from 20% to 65% in 35 min.
4. The UV detection wavelength was 280 nm, the injection volume was 10 L, and the column temperature was set at 35 °C.
5. Spectra manager CFR software was used for spectra acquisition and analysis. Spectra with high noise are usually removed in analysis.
6. The parameters of the two commonly used hyperspectrometer (ImSpector V10E and ImSpector N17E) are shown in Table 1.
7. The probe is positioned above the surface of the sample at a distance of 15 mm and the sample view angle of the probe is 30°. The light source is positioned at a distance of 150 mm and at an angle of 30°from the surface of the sample.
8. Scrubbing the salt tablets of FT-IR spectrometer from the inside out, and avoid getting too much of organic solvents, such as acetone.
9. Before the experiment, potassium bromide (KBr; SCR, Shanghai, China) was dried in an oven at 105 °C for 4 h and cooled in a desiccator.

Table 1
The parameters of ImSpector V10E and ImSpector N17E

Parameter	ImSpector V10E	ImSpector N17E
Spectral range	400–1000 nm	900–1700 nm
Spectral resolution	2.8 nm	5 nm
CCD pixel	672 (Front length)×512 (Band number)	320 (Front length)×256 (Band number)
Exposure time	0.01–1000 ms	0.01–500 ms
Sampling interval	0.59 nm	0.65 nm
Effective slit length	14.2 nm	14.2 nm
Focal length	23 mm	22 mm
Slit width	30 μm	30 μm

10. In theory, the particle size of the grinding of FT-IR sample should be smaller than the wavelength of its infrared light in order to avoid the generation of dispersion spectra. Pay attention not to absorb moisture during the grinding process and do not exhale towards the sample.
11. When making FT-IR samples, pay attention to opening and closing the sample chamber gently, and do not exhale facing the sample chamber, as it can make the background absorb well.
12. Hand washing and drying are important when collecting the FT-IR spectra of tea samples.
13. Some regression algorithms are usually applied to establish determination models: partial least squares (PLS), radial basis function neural network (RBF-NN), least-squares support vector machine (LS-SVM), and Gaussian process regression (GPR) [15, 16]. PLS is one of the reliable analytical tools for modeling and has a wide application in multivariable data analysis and regression [17]. RBF-NN has a strong ability for nonlinear fitting, which can be applied to approximate functions. LS-SVM is an optimized algorithm based on standard SVM, and has the capability for linear and nonlinear multivariate calibration and solves the multivariate calibration problems in a relatively fast way [18]. GPR is a machine learning method based on Bayesian theory and statistical learning theory.
14. Spectra often contain some undesired effects (sample physical characteristics, instrument response and working environment) which are irrelevant to the substance studied and would decrease the accuracy of the calibration models. Thus, mathematical treatments are often applied to the spectra to remove the interferences of these disturbing information before modeling [19, 20]. These pretreatments were baseline correction (BC), standard normal variate (SNV), multiplicative scatter correction (MSC), smoothing (SM), first derivate (1st DER), second derivate (2nd DER), Savitzky-Golay (SG) smoothing, wavelet transform (WT), detrending (DET), and normalization (NOR). These methods are widely used in preprocessing of spectral data.

    Three typical preprocessing methods are introduced:

    BC is mainly used to eliminate the interference of instrument background or drift on spectral signals. Existing baseline correction algorithms include polynomial fitting, segmented polynomial fitting, wavelet transform, etc. Among them, the step of piecewise polynomial fitting baseline correction is to divide the spectral curve into several intervals, and the width of the interval is used as the window width for analysis. The spectral curve characteristics of each window are processed

separately. Usually, the minimum value of the spectral curve of each window is selected as the feature point, and then the feature points in each window are connected with straight lines, and the connected broken lines are fitted into an approximate linear relationship. The final fitted curve is considered as the baseline, and the corrected spectral data is obtained by subtracting the baseline from the original spectral curve.

SNV is used to correct spectral errors caused by scattering between samples, and is widely used in the preprocessing of reflection spectral data of solid agricultural products. The basic calculation idea of the algorithm is: assuming that the reflection intensity of each wavelength point in each spectrum should conform to a certain distribution (such as normal distribution), based on this assumption, each original spectral data will be standardized and normalized.

MSC is similar to SNV, which eliminates the effects of uneven particle distribution and particle size by correcting the scattering of each spectrum. MSC has a wide range of applications in solid-state diffuse reflectance spectroscopy. This algorithm assumes that each spectrum has a linear relationship with the "ideal" spectrum, but the true "ideal" spectrum does not exist. Therefore, the average spectrum of the sample is considered as the "ideal" spectrum.

15. Spectral data have many variables, among which there is only a small amount of useful information and most of them are irrelevant and collinear variables. The commonly used approach to solve this problem is to select useful variables from many variables through the characteristic wavelength selection methods. There are some selection algorithm of feature wavelength, including competitive adaptive reweighted sampling (CARS), least square support vector regression (LSSVR), shuffled frog leaping algorithm (SFLA), successive projections algorithm (SPA), which are used to find the variables with the lowest redundancy from datasets.

CARS algorithm is a common method for selecting spectral characteristic variables. It uses MSC method to randomly select a certain proportion (generally 80%) of all samples as the modeling set, establishes the PLSR model, then uses Exponential decay function to remove variables with smaller weight values, and uses adaptive reweighting sampling algorithm to filter variables with larger weight, and finally selects the subset of the PLSR model with the smallest root mean square error of cross validation (RMSECV).

Least square support vector machine (LSSVM) is a data analysis method combining least squares and support vector machine. Similar to support vector machine, the basic idea of this method is to map low-dimensional raw data nonlinearly to

high-dimensional space, but the difference is that inequality constraints are used instead of equality constraints. In high-dimensional linear space, minimizing the loss function to obtain a linear fitting function. The parameters of this fitting function are obtained by solving a finite dimensional quadratic programming problem in the dual space. Therefore, this method is more efficient than support vector machines.

SFLA is a heuristic population evolutionary algorithm with high computing efficiency and excellent global search ability. The algorithm calculates the selected probability of each variable by simulating a Markov chain obeying the steady distribution in the model space, so as to select the characteristic variable. SFLA is used in conjunction with the PLSR method when selecting feature bands for spectra, to determine whether the variable needs to be removed during each iteration based on the absolute value of the regression coefficient in the PLSR model.

SPA is a forward variable selection algorithm that minimizes the collinearity of variable space. This method has a direct idea for the selection of characteristic bands, that is, starting from a band, forward cycling step by step. Each cycle calculates the projection of the selected band vector on the unselected band vector, and introduces the band with the largest projection distance to the set of characteristic bands.

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Chapter 2

Comprehensive Analysis of Pistachio (*Pistacia vera* L.) Hull Phenolics by High-Performance Liquid Chromatography-Diode Array Detection-Tandem Mass Spectrometry

Sevcan Erşan

Abstract

Pistachio hull, the by-product of pistachio processing, is a plant source rich in phenolic compounds with diverse biological activities that may promote human health. Here, we describe the high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD) and tandem mass spectrometry (MS/MS)-based procedure for comprehensive analysis of phenolic compounds from pistachio hull. The analytical approach encompasses sample preparation, phenolic extraction, HPLC separation, DAD and MS detections, data analysis, and interpretation. Additionally, method validation parameters are presented, ensuring the accuracy and reliability of the analytical process. The described methodology enables quantitative determination of a wide range of phenolic compounds from pistachio hull, including flavonoids, anacardic acids, and gallotannins.

Key words Nut, Waste, Polyphenol, Gallic acid, Tannin, Bioactive, HPLC-DAD-MS/MS, Mass spectrometry profiling, Fragmentation

1 Introduction

Pistachio hulls are rich in phenolic compounds associated with various potential health-promoting effects [1]. The pistachio hull phenolics were linked to anti-inflammatory [2], anti-obesity [3], and anti-cancer properties [4, 5], suggesting their potential in preventing certain diseases. Pistachio hull and their extracts were considered functional ingredients in various food products [6, 7]. They have been explored as natural alternatives for synthetic antioxidants commonly used in the food industry [8–12], highlighting their potential as techno-functional food ingredients. Hence, pistachio hull and their extracts have immense potential for use in the food, pharmaceutical, and biomedical industries.

Mostafa Gouda et al. (eds.), *Plant Chemical Compositions and Bioactivities*, Methods and Protocols in Food Science,
https://doi.org/10.1007/978-1-0716-3938-2_2,

Further research is required to fully understand the extent of these health benefits and technological functions and their association with pistachio hull phenolics. A comprehensive phenolic analysis is essential to elucidate the contribution of individual phenolic components to biological activities, ascertain optimal dosages for therapeutic effects, and standardize the phenolic compositions of extracts. This is particularly crucial as poorly characterized extracts often lead to highly variable, unpredictable, and sometimes even partly controversial biological activities.

The phenolic compositions can be accurately assessed with high specificity using high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) and mass spectrometry (MS). This approach overcomes the limitations of commonly used nonspecific spectrophotometric methods, such as the Folin-Ciocalteu and activity-based assays. In the HPLC-DAD-MS/MS system, HPLC-separated analytes are ionized in an electrospray ionization (ESI). Subsequently, MS detects the charge-to-mass ratio (m/z) of precursor or fragment ions. The additional DAD provides ultraviolet-visible light (UV/Vis) spectra characteristic of each class of phenolic compounds. Consequently, phenolic compound identification can be reliably accomplished by comparing the LC retention times, UV/Vis spectra, and MS spectra with those of standard compounds [13].

When standard compounds are commercially unavailable, and their preparation at high purity is impractical, phenolic compound identification can be achieved by comparing the HPLC-DAD-MS/MS data with that from earlier research and evaluating UV and MS data and the expected fragmentation patterns. In our previous comprehensive study, we employed the HPLC-DAD-MS/MS approach following the described methodology and provided detailed insights into 66 individual phenolic compounds from pistachio hull with minimal reliance on standard compounds [13]. Among the three major phenolic classes present in pistachio hull, namely gallotannins, flavonoids, and anacardic acids, the most prevalent compounds were anacardic acids, gallic acid, monogalloyl glucose, pentagalloyl glucose, quercetin galactoside, and quercetin glucoside (Fig. 1). Subsequently, 58 phenolic compounds quantitated in the pistachio hull extracts utilizing six standard compounds [14], considering that structurally related phenolic compounds exhibit similar UV/Vis absorbances at their specific detection wavelengths when aromatic, or aglycon, parts of the phenolic structure resemble each other or remain the same [15]. As a result, the phenolic composition of pistachio hulls, determined by HPLC-DAD-MS/MS, ranged from 61.2 to 100 g/kg dry matter (DM), underscoring them as good sources of various individual phenolic compounds [14].

This chapter outlines a workflow for HPLC-DAD-MS/MS analysis of the pistachio hull phenolics (Fig. 2), extending from

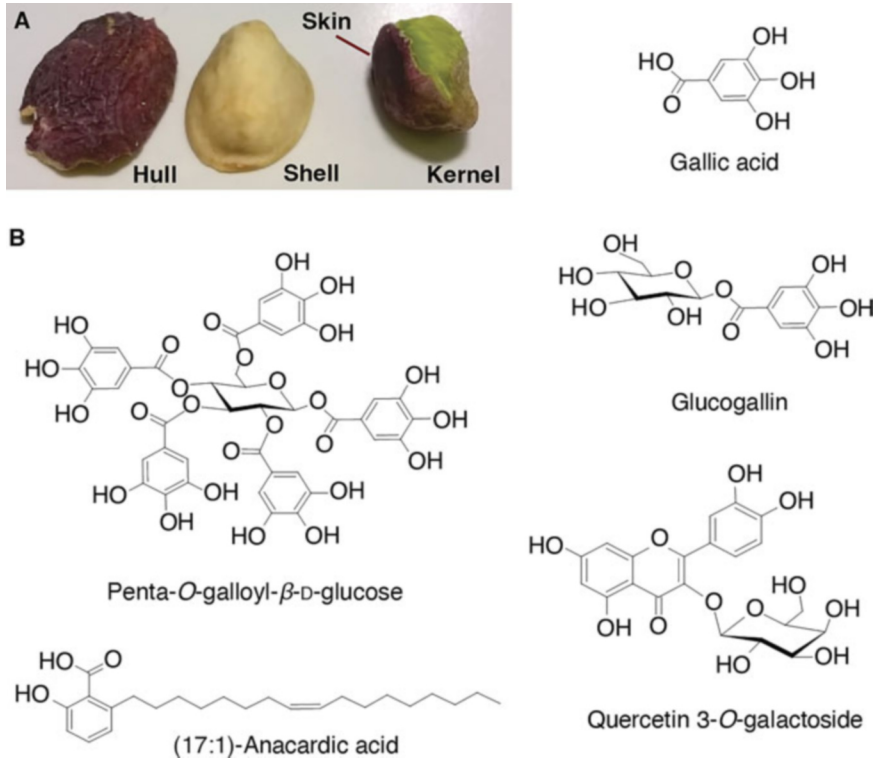

Fig. 1 Pistachio hull, the primary by-product of pistachio processing, constitutes the outer layer of pistachio drupe. The remaining components of the pistachio drupe include lignocellulosic hard shell and edible green kernel covered by red skin (**a**). Representative phenolic structures from pistachio hull (**b**)

our studies [13, 14]. The presented method warrants future research into the phenolic composition of pistachio hulls, including investigations across different varieties cultivated under diverse environmental conditions, samples collected from technologically differing processing units, and for the standardization of derived extracts. This approach allows a more comprehensive understanding of the biological activities linked to pistachio hull phenolics, thereby promoting their potential applications.

2 Materials

2.1 Equipments

1. Analytical balance, 0.1 mg sensitivity.
2. Calibrated pipettes, 1 mL, and 100 or 300 μL volumes.
3. Freeze-dryer (*see* **Note 1**).
4. Analytical grinding mill.
5. Infrared moisture analyzer (*see* **Note 2**).
6. Mili-Q water system.

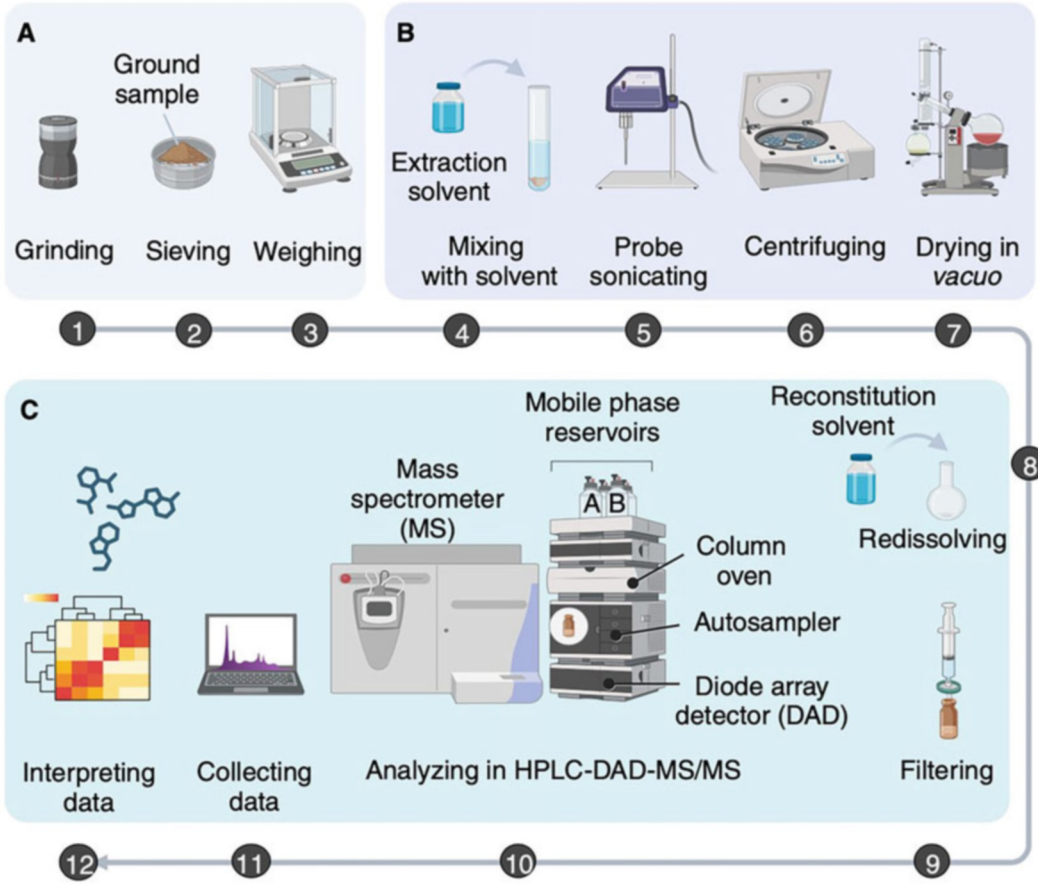

Fig. 2 Workflow for the analysis of pistachio hull phenolics. (**a**) Sample preparation; (**b**) Phenolic extraction; (**c**) HPLC-DAD-M/MS analysis and data processing. (Created with BioRender.com)

7. Vortex mixer.
8. Ultrasonic probe sonicator.
9. Ultrasonic bath (optional) (*see* **Note 3**).
10. Magnetic stirrer plate.
11. Centrifuge, compatible with available glass test tubes.
12. Rotary evaporator.
13. HPLC system (with a quaternary pump, an autosampler, and a vacuum degasser) coupled with a diode array detector (DAD) and a mass spectrometer (e.g., Esquire 3000+ ion-trap mass spectrometer, Bruker Daltonics, Bremen, Germany) fitted with an electrospray ion (ESI) source. Instrument control and data acquisition softwares (e.g., Chromquest and Esquire Control).
14. (Optional) A high-resolution mass spectrometer (e.g., micrOTOF-Q mass spectrometer, Bruker Daltonics, Bremen, Germany or Q Exactive Plus high-resolution mass spectrometer, Thermo Fisher Scientific, Bremen, Germany) (*see* **Note 4**).

2.2 Reagents

1. Water, LC-MS grade.
2. Methanol, LC-MS grade.
3. Formic acid, 98–100%.
4. Hydrochloric acid (HCl), 37%.
5. Liquid nitrogen.
6. Nitrogen gas (*see* **Note 5**).
7. Analytical standard compounds at the highest available purity (*see* **Note 6**): Gallic acid; Protocatechuic acid; β-Glucogallin (1-*O*-galloyl-β-D-glucopyranose); Penta-*O*-galloyl-β-D-glucose; (15:0)-Anacardic acid; Quercetin 3-*O*-galactoside; Quercetin 3-*O*-glucoside; Quercetin 3-*O*-glucuronide; Myricetin 3-*O*-galactoside; Cyanidin 3-*O*-β-D-galactopyranoside.

2.3 Pistachio Hull Samples

1. Pistachio hull: fresh or dried whole pistachio drupes containing hull, shell, skin, and kernel (Fig. 1) or waste stream collected from the processing unit (*see* **Note 7**).
2. Store pistachio hull samples at −20 °C until analysis.

2.4 Supplies

1. Sample storage bottles.
2. Weighing paper.
3. Micro spatulas.
4. Lab spoon or spatula.
5. Microcentrifuge tubes, 1.5 or 2 mL volume.
6. Pipette tips, compatible with calibrated pipettes.
7. Glass reagent bottles, 100 mL volume.
8. Glass test tubes (5 or 10 mL volume) with stoppers or caps, compatible with available centrifuge setup.
9. Glass pipettes, 1 and 10 mL volumes.
10. Pipette pumps, compatible with glass pipettes (**item 9**).
11. Magnetic stirring bars (*see* **Note 8**).
12. Pasteur pipettes and compatible pumps (*see* **Note 9**).
13. Round bottom evaporation flasks, 25 or 50 mL, and flask supports (*see* **Note 10**).
14. Glass graduated cylinders, 50, 100, and 1000 mL volumes.
15. LC solvent bottles, 4 of 1 L volume (*see* **Note 11**).
16. Amber LC glass vials with caps, 2 mL volume (*see* **Note 12**).
17. Vial inserts, 100 or 300 μL volume, compatible with glass vials (**item 16**).
18. Plastic or glass syringe, 1 mL volume.
19. Regenerated cellulose syringe filters, 0.45 μm pore size.