Determination of oleanolic acid in natural products, using radix Achyrantes bidentatae as a marker sample

YEUNG Ming Fai

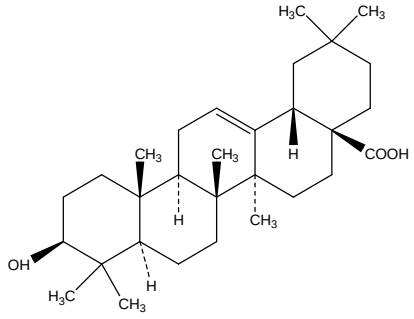
Keywords: Oleanolic acid (OA); High performance liquid chromatography (HPLC); Radix Achyrantes bidentatae

Abstract

A method for determination of Oleanolic acid (OA) from radix Achyrantes bidentatae (RAB) was developed, optimized and validated. In addition, a novel approach for determination of OA from natural products was developed by generalization of method of determination of OA from RAB.

Introduction

Oleanolic acid (OA), a common phytochemical in CHM which is a naturally ocurring triterpene present in various leaves, roots and bark of various plants. Its structure was definitively established by Ruzicka in 1946. (Figure 1)



Structural formula of Oleanolic acid (OA)

Triterpene refers to a particular type of molecular structure that has a four- or five-ring, planar-base molecule containing 30 carbon atoms. It can link to sugar molecules to form saponins. When saponins are consumed, the sugar molecule is usually cleaved off by enzymatic action either in the gut or in the blood stream.

There are different techniques for determination of OA in natural products. They include:

High performance liquid chromatography (HPLC)

Liquid chromatography is a type of chromatography, which has been used for the separation and identification of compounds. The use of HPLC can increase the separation efficiency of the method. In HPLC, liquid mobile phase is employed and the separation is based on the interaction of analytes between the mobile phase and stationary phase coated on the supported solids. Since a large number of organic compounds are either insufficient volatile or unstable at high temperature, HPLC becomes the first choice rather than gas chromatography.

An HPLC instrument requires a high pressure pump and a supply of mobile phase, a column containing a high efficiency stationary phase, an injection unit for introducing samples to the column, an online detector and some method of displaying the detector signal. Markers were used for the quality control of that herb.

Standard solution mixture of marker compound was prepared with known concentration. Then HPLC chromatogram was obtained and peak area was determined. After that, the HPLC chromatogram of standard decoction was obtained. The concentration of marker compound in standard decoction was calculated as follow and their values were compared.

$$\frac{\text{Conc. of marker in sample}}{\text{Conc. of marker in standard solution}} = \frac{\text{peak area of marker in sample}}{\text{peak area of marker in standard solution}}$$

The peak areas of marker in samples and standard solution can be obtained in chromatogram and the concentration of marker in standard solution were known. Therefore, concentration of each marker in each sample can be calculated.

HPLC is broadly used to analyze the components of ingredients. There are other alternatives: electrochemical method (such as titration), thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE) and inductively-coupled plasma-mass spectrometry (ICP-MS). The comparisons of the methods with HPLC method were tabulated (Table 1).

	Electrochemical method such as Redox titration	**TLC**	**GC**	**CE**	**ICP-MS**
Determinants	Oxidizable and reducible compounds and ions	Compounds and ions	Volatile organic compounds which are thermally stable	Charged ions	Metallic elements
Accuracy	Lower	Lower	Comparable	Lower	Comparable
Precision	Lower	Lower	Comparable	Lower	Comparable
Selectivity	Lower	Lower	Higher	Higher	Higher
Sensitivity	Lower	Lower	Higher	Higher	Higher
Samples recollection	May be	Can be	Cannot be	Can be	Cannot be
Set-up Cost	Lower	Lower	Comparable	Comparable	Higher
Operation cost	Lower	Lower	Comparable	Lower	Higher

Table 1. Comparison on different techniques for determination of ingredients in natural products.

Electrochemical method

In titrimetric method, ingredients in natural products are extracted by different solvents and methods. Color indicator was added to the end-point until the change of color mixture. Content of ingredient can be calculated by the volume of indicator added by:

Mass of ingredient
= Molarity of indicator x Volume of indicator added x Molar mass of indicator x Ratio of number of mole of indicator to number of mole of ingredient in the balanced equation

Apart from the colorimetric method, Redox titration, potentiometry, electrogravimetry, coulometry and voltametry can be used for analyzing samples. Samples analyzed by Redox titration are restricted to be oxidizable and reducible.

Advantage over HPLC

Lower instrumental setup cost

Set-up cost for a simple titration is no more than $1000, whereas the set-up cost for HPLC is about $100,000 - $150,000. Simple apparatus are used such as burette, pipette, conical flask, etc. In HPLC, simple apparatus are used for

extraction. But regenerated cellulose filters, column and HPLC grade solvent are expensive.

Lower operation cost

Operation cost of HPLC includes a large amount of HPLC-grade organic solvent as mobile phase which are expensive.

Weaknesses over HPLC

Limited types of determinants

Separation of compounds and determining the end-point volume by human effort

Titration is suitable for detecting one group of compounds, such as total flavonoids in natural products. For detecting single compounds, several separation and purification steps are needed. This requires a lot of human time and effort to obtained purified compounds.

Low accuracy and precision

Accuracy and precision are low due to human errors and measurement uncertainty in the extraction and measurement processes as compared to HPLC.

Low sensitivity and selectivity

Sensitivity is low because of the poor separation efficiency in the extraction processes. Selectivity is low due to chemical interference. An ingredient in natural products is required to be high enough to give a visual color change during titration. Low concentration will lead to higher human errors and measurement uncertainty in which accuracy and precision will be lowered. For the HPLC detection, detection limit and quantitation limit can be as low as μg to ng levels.

Difficult to recollect samples after analysis

Some chemical reactions in the titration process are not reversible. If reversible, purification steps are needed. But in HPLC, purified compounds in the mixture can be recollected after analysis.

Thin layer chromatography (TLC)

In TLC method, ingredients in natural products are extracted by different solvents and methods and then spread out on a TLC plate by different solvent systems using chromatographic method. Sometimes, coloring agents are spread to color certain compounds. Ultraviolet-visible (UV-Vis) light with different wavelength is aided to see the spots of compounds on TLC. The contents of compounds are determined by area and density of spots on TLC, compared with calibration curve of standard compounds. UV-Vis spectroscopic method can be applied for the detection of dissolving the determinant's spot on TLC plate. In UV-Vis spectroscopic method, UV-Vis light is emitted from a source (deuterium for UV and tungsten for Visible) and the sample absorbs light in certain wavelengths. The light passed out from sample is detected by detector in UV-Vis spectrometer. The contents of compounds are determined by peak areas of ingredient in UV-Vis spectrum, compared with calibration curve of standard compounds. .

Advantage over HPLC

Lower instrumental setup cost

Set-up cost for a simple TLC determination method is no more than $50000, whereas the set-up cost for HPLC is about $100,000 - $150,000. Apparatus such as TLC plate and UV lamp/ UV-Vis spectrometer are used. In HPLC, simple apparatus are used for extraction. But regenerated cellulose filters, column and HPLC grade solvent are expensive.

Lower operation cost

Operation cost of HPLC includes a large amount of HPLC-grade organic solvent as mobile phase which are expensive.

Weaknesses over HPLC

Low accuracy and precision

Accuracy and precision are low due to human errors and measurement uncertainty in the extraction and measurement process.

Low sensitivity and selectivity

Sensitivity and selectivity are low because of the poor separation efficiency and higher detection limit in TLC separation as compared to HPLC.

Gas chromatography (GC)

Gas chromatography is a separation method composed of a gas mobile phase (such as helium) and a stationary phase.

Samples that are volatile or thermally stable can be separated using this method. A mixture of analytes is introduced into the mobile phase and is carried through the system. As the mobile phase passes over and through the stationary phase, the components of mixture equilibrate or partition between the two phases, resulting in differential migration rates through the system. Ultimately, different components of the mixture will be separate in time. Gas chromatography coupled with mass spectrometry (GC-MS) is widely used for the identification and quantitative analysis of organic compounds. The high separation efficiency of GC plus the sensitive and specific detection using the MS make it one of the most powerful techniques in modern analytical instrumentation. The retention time and peak areas for different concentration of organic compounds and samples were obtained. The presence of compounds was ensured by library search and the retention time in the GC chromatograms. Then calibration curves were plotted, using the ratio of area of standard to area of internal standard. The slope and the r value (coefficient of correlation) for each calibration curve were reported and the amount of compounds in the spiked sediment sample can be determined from the calibration curve. The amount of compounds in sample of natural products was reported and the percentage recoveries of the compounds in the experiment can be calculated by:

$$\frac{\text{Conc. of marker in sample}}{\text{Conc. of marker in standard solution}} = \frac{\text{peak area of marker in sample}}{\text{peak area of marker in standard solution}}$$

Advantage over HPLC

High sensitivity and selectivity

Sensitivity of GC is higher than HPLC. Typical limit of detection (LOD) of HPLC with diode-array detector (DAD) is µg to ng level. LOD for GC with MS as detector is ng to pg level.

Separation efficiency of GC is higher than HPLC. From figure 2.19(b), capillary GC has $n_{TP} \fallingdotseq$ (100 à 200) x 10^3 with min. $\alpha \fallingdotseq 1.03$ and HPLC packed columns has $n_{TP} \fallingdotseq$ (5 à 10) x 10^3 with min. $\alpha \fallingdotseq 1.10$

From equation,

$R_s = (n_{TP}^{1/2}/4)\,[(\alpha-1)/\alpha]\,[k_b/(1+k_b)]$ [23, 24], whereas k_b is the retention factor of the slower moving species and α is the selectivity factor. Therefore, R_s for HPLC < GC [25].

Weaknesses over HPLC

Limited types of determinants

Only volatile organic compounds which are thermally stable can be used for GC detection. Samples are heated up in the injector before running the capillary. Non-volatile compounds may block the injector. Compounds which are thermally unstable will be degraded.

Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is currently one of the most important techniques for separation of charged analytes. Three separation science technologies converged in the development of commercial CE instruments. The fused silica capillaries which revolutionized gas chromatography a decade earlier proved to be ideally suited as the separation chamber for CE. The high-sensitivity absorbance detectors developed for HPLC were easily adaptable for CE with modifications in the optical components to permit on-tube detection. The broad range of separation modes achieved by labor gel electrophoresis techniques could now be automated. Combining the advantages of these three powerful separation technologies has produced the new analytical tool of CE, providing the highest levels of resolution and sensitivity with a complete spectrum of separation applications.

CE employs the separation mechanisms of conventional electrophoresis in a capillary format. Separated components are identified by on-line detection during the analysis.

A general CE system usually comprises a detector, dual high voltage power supplies, and an electrophoresis compartment containing a capillary connecting two buffer reservoirs with high voltage electrodes. Molecules are

separated as electrical force drives them at different rates through a capillary filled with electrolyte. Separated sample components are monitored by the detector as they migrate through a segment of the capillary, and the detector signal is displayed as peaks on an electropherogram. In contrast to HPLC, where all components migrate through the detector flow cell at equal velocity, the integrated peak areas in a CE electropherogram are functions of both analyte concentration and migration velocity.

All CE separations are performed using fused silica capillaries which are externally coated with a polymer such as polyimide to improve their mechanical strength. Since the polyimide cladding is not UV-transparent, a segment of the cladding must be removed to provide a monitor point for UV detection. This increases the fragility of the capillary. Incorporation of the capillary into a cartridge eliminates the risk of capillary breakage and enables the capillary to be pre-aligned with a microfocusing lens system for sensitive and stable detection. The cartridge format also enables efficient temperature control of the capillary by passage of a temperature-stabilizing liquid across the external surface of the capillary. Capillary thermo-stating is critical to achieving good precision of analyte migration time and peak area for quantitative analysis.

Many small ionic molecules have been separated by CE, including nucleosides and nucleotides, amino acids, vitamins, and ionic drugs. The addition of a chiral selector to the buffer can allow separation of stereoisomers, and CE may prove to be superior to HPLC for characterization of chiral drugs and their metabolites.

Advantages over HPLC

Rapid micro-volume analysis

Rapid micro-volume analysis is one key advantage of CE over HPLC. Since only nanoliters of sample are consumed in each analysis, literally hundreds of CE runs can be performed from the microliter sample volumes typically consumed for a single HPLC injection. As CE separation technology has become apparent that tremendous flexibility in separation selectivity can be achieved by simple changes in the composition of the electrophoretic buffer. This enables a single capillary to be used to obtain separations based on a wide range of molecular properties (e.g. size, charge, chirality, hydrophobicity). Separation techniques can be quickly developed and optimized by rapid scouting experiments with simple buffer changes, shortening method development time from a matter of weeks or months to a few hours or days.

Reverse-phase HPLC separates according to hydrophobic interaction of the analyte with the stationary phase. Therefore, polar and charged compounds are often not very well analyzed.

Higher selectivity and sensitivity

The fused silica capillaries which revolutionized gas chromatography proved to be ideally suited as the separation chamber for CE. The separation efficiency and selectivity are thus higher than HPLC. The high-sensitivity absorbance detectors developed for HPLC were easily adaptable for CE. Because of the higher separation efficiency with less peak broadening as compared with HPLC, the sensitivity is thus higher in CE than HPLC. Detection limit in CE can be up to ng levels

Lower operating cost

CE does not have the problems of HPLC solvent disposal, while HPLC requires a lot of HPLC-grade solvent and the disposing charges are expensive. The separations are also more robust and cost effective compared with HPLC where chirally selective columns are often expensive.

Lower solvent interference

Unlike HPLC solvents, most CE buffers do not exhibit low-UV absorbance

Weaknesses over HPLC

Lower accuracy and precision

The repeatability and reproducibility are poorer as compared to HPLC. It is because electroosmotic flow in CE is difficult to be controlled. A lot of factors which are in a complicated matrix affecting the electroosmotic flow. Therefore, repeatability and reproducibility of retention time in CE is quite poorer as compared to HPLC.

Limited types of determinants

Only charged analytes can be analyzed by CE.

Inductively-coupled plasma – mass spectrometry (ICP-MS)

Inductively coupled plasma- mass spectrometry (ICP-MS) is a hyphenated technique of linking mass spectrometry (MS) to inductively coupled plasmas (ICP). It is widely used in determining the element composition of different types of samples. ICP acts as an ion source for MS. The ICP quartz torch is a device that produces plasma – a fireball of atoms, ions and electrons interacting at very high energies with temperatures up to 10000K. As the argon flows through the inner tube of the torch, it is seeded with free electrons from a discharge coil. As the charged particles are forced to flow in a closed annular path, an eddy current of electrons and cations is formed. Further ionization is produced as these rapid moving ions and electrons collide with other argon atoms, leading to high thermal energy as they meet resistance to their flow. A second stream of argon gas passes through the outer tubes of the torch to keep the torch cool, and to provide a gas flow to center and stabilize the plasma. This technique, with the high efficiency of atomization and ion formation of the ICP is complemented by the specific and sensitive multi-element detection capability offered by MS.

For analysis of samples, it is passed into the plasma stream in a solution usually via an HPLC column. The sample is introduced as an aerosol through the center tube of the torch into the plasma by means of nebulizer connected to a spray chamber, which separates and removes the larger droplets of the aerosol. As the analyzer region of an ICP-MS is maintained at low pressure, the ions produced in the plasma are drawn into the mass analyzer via a pressure differential. The ions enter through the sampler and skimmer cones and are focused into the mass analyzer via a series of lenses. As in the other types of MS analyzers described earlier, the ions being analyzed by ICP-MS are separated on the basis of their m/z ratio.

Advantages over HPLC

Powerful tools for element composition analysis

It is widely used in determining the element composition of different types of samples. HPLC is difficult to analyze molecules in element level, even coupled with MS detector.

Higher sensitivity and selectivity

Separation of ions in the quadrupole mass analyzer is according to m/z ratio. The selectivity is thus higher than the column separation in HPLC. Detection limit in ICP-MS can be up to ng-pg levels which HPLC-DAD has detection limit of µg to ng levels. Therefore, ICP-MS is a powerful choice for trace elemental analysis.

Disadvantages over HPLC

Higher instrumental setup cost

ICP-MS costs about $1,000,000 and HPLC costs about $100,000 - $150,000.

Higher operation cost

Maintenance costs for ICP-MS are also higher as compared with HPLC

Limited determinant detection

Molecules are destruct under high temperature in ICP torch. Only elements can be analyzed in ICP-MS.

After the comparison, choosing techniques for determination of ingredients in natural products depends on the nature of ingredients and availability of instruments. HPLC was the most promising technique because of its high precision, high accuracy, low LOD, low LOQ and suitability for wide variety of compounds. Therefore, HPLC was chosen as the techniques for determination of OA in natural products.

RAB was chosen as marker sample because OA cannot be extracted by normal alcoholic extraction but can be extracted by acid hydrolysis after alcoholic extraction. To develop method of OA extraction from natural products, the method needs to have the ability to extraction

Review

Flow chart of extraction processes for the determination of OA in RAB done in 1994 was described in figure 2 [1]. Procedures of acid hydrolysis were not optimized resulting in underestimation of OA content.

Flow chart of extraction processes for the determination of OA in RAB done in 1997 was described in figure 3 [2]. Sample powder was directly refluxed with 70% EtOH and 10% HCl for 2 hours and the extract was filtered. Solvent was removed by steam evaporation and then transferred to V-flask by MeOH. The volume was made up with MeOH prior to HPLC analysis. However, the conditions for extraction and acid hydrolysis were not optimized and it was difficult to dissolve the entire residue in MeOH after steam evaporation.

Flow chart of extraction processes for the determination of OA in RAB done in 2000 was described in figure 4 [3]. Powdered RAB sample was acid hydrolyzed by HCl through heating under reflux for 2 hours. The mixture was filtered and washed with 95 % EtOH. The filtrate was heating under reflux for 2.5 hours. The mixtrure was filtered and the filtrate was mixed with water and chloroform for LLE. The chloroform layers were combined and rotary evaporated. EtOH and ether (2:3) was added to dissolve the supernatant. OA content was determined by TLC method. However, TLC determination has poor accuracy and precision as compared with HPLC. Also, the acid hydrolysis process was not optimized in this method. Complex extraction process was another problem.

Another research was done in 2001 and flow chart of extraction procedures was described in figure 5 [4]. The sample was hot extracted by 70% EtOH for 1 hour and then undergone centrifugation. A portion of clear solution was heated with HCl for 3 hours and then undergone rotary evaporation. LLE with chloroform was used in the extraction procedures. The mixture was filtered and then rotary evaporated to remove the solvent. Finally the mixture was made up with MeOH prior to HPLC analysis. This research focuses on using orthogonal design to optimize the extraction of triterpene saponins with OA as aglycone. However, the conditions for acid hydrolysis are not optimized.

Another research was done in 2003 and the extraction processes was described in figure 6 [5]. Powdered RAB sample was acid hydrolyzed by 5 % HCl through heating under reflux for 6 hours. The mixture was filtered and incubated for 2 hours to dryness. Water and chloroform were added for LLE. The chloroform layers were combined and rotary evaporated. EtOH and chloroform (3:2) were added to dissolve the supernatant. OA content was determined by TLC method. However, TLC determination has poor accuracy and precision as compared with HPLC. Also, the acid hydrolysis procedures were not optimized in this method. Complex extraction procedures were another problem.

Another research was done in 2005 and the extraction procedures were described in figure 7 [6]. Powdered RAB sample was hot extracted by 70% EtOH for 1 hour and then undergone centrifugation. A portion of clear solution was heated with 20 % HCl for 3 hours and then undergone rotary evaporation. LLE with chloroform was used in the extraction procedures. The mixture was filtered and then rotary evaporated to remove the solvent. Finally the mixture made up with 90% MeOH prior to HPLC analysis. This research focuses on using orthogonal design to optimize the extraction of triterpene saponins with OA as aglycone. However, the conditions for acid hydrolysis were not optimized.

Figure 2 **Flow chart of extraction processes for the determination of OA in RAB done in 1994.**

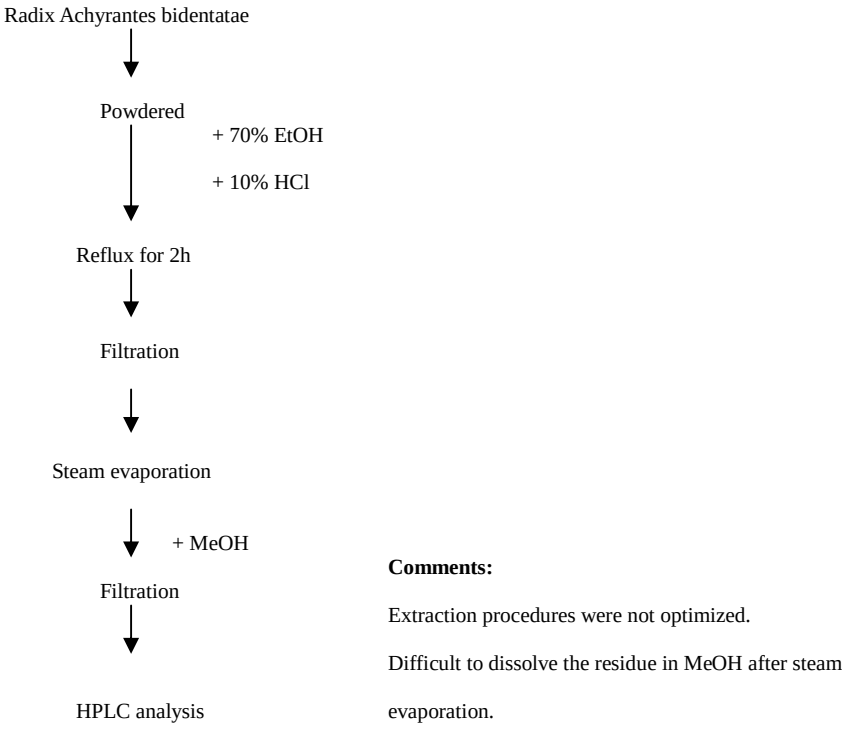


Figure 3 **Flow chart of extraction processes for the determination of OA in RAB done in 1997.**

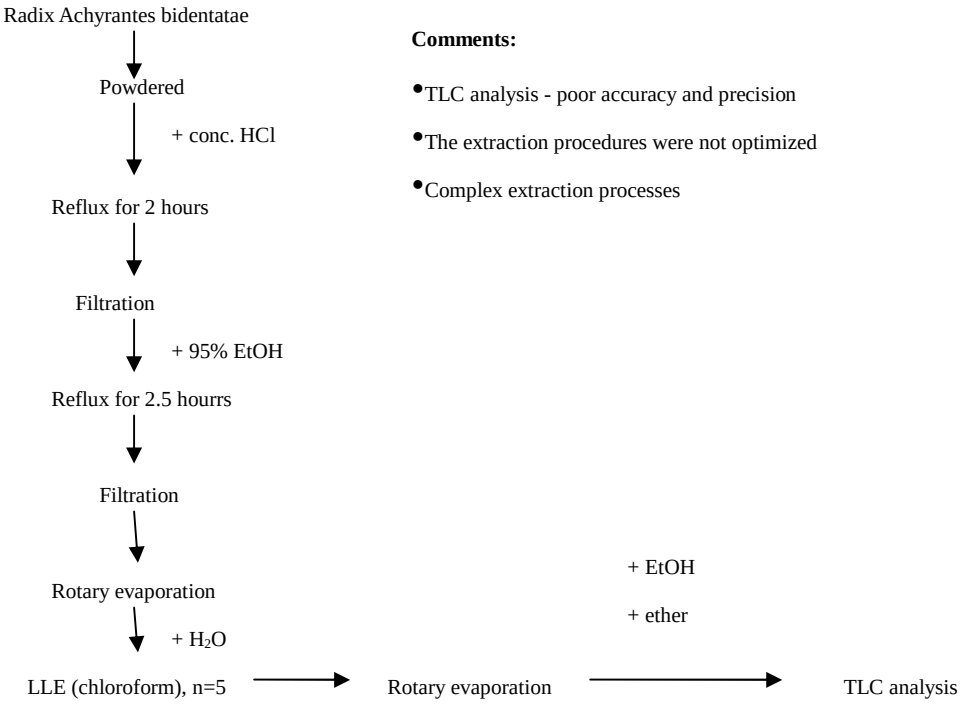


Figure 4 **Flow chart of extraction processes for the determination of OA in RAB done in 2000.**

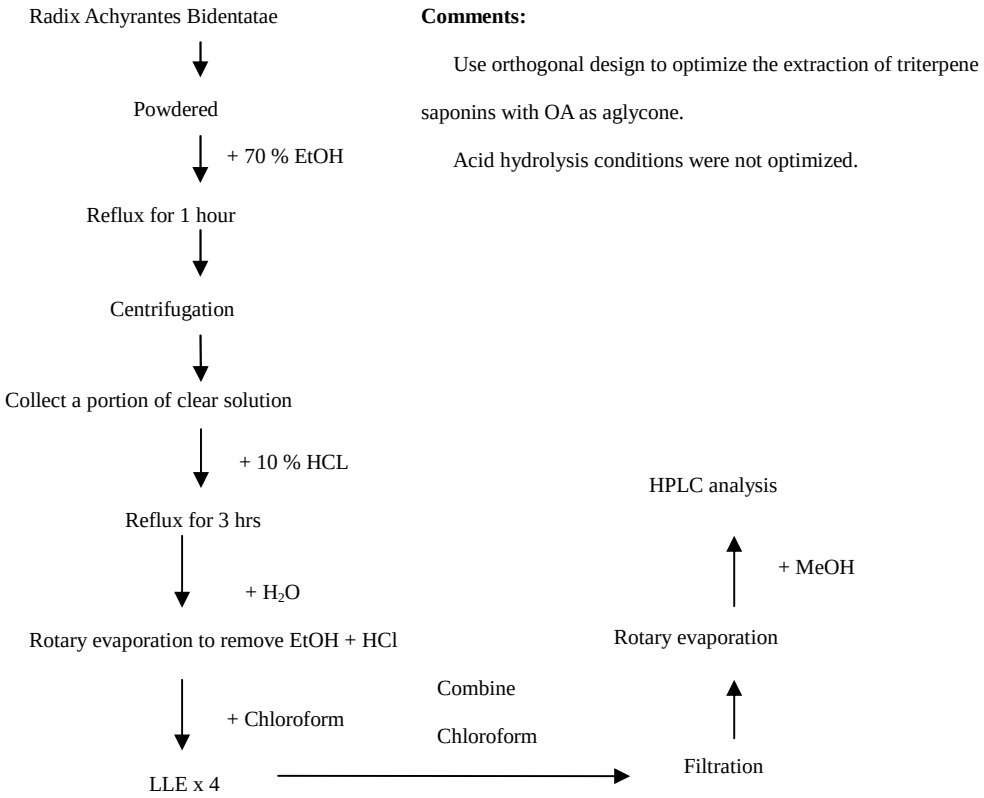


Figure 5 **Flow chart of extraction processes for the determination of OA in RAB done in 2001.**

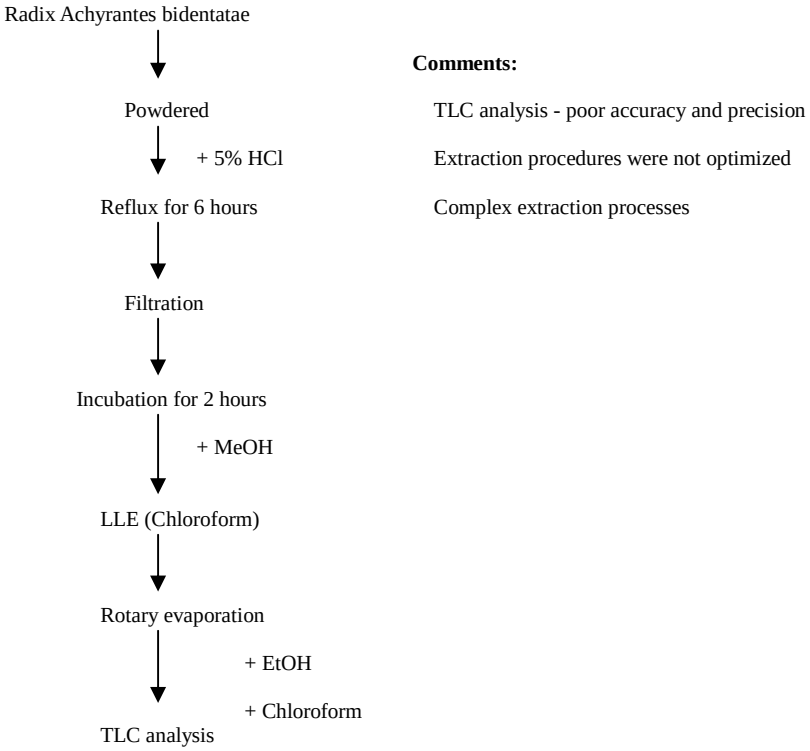


Figure 6 **Flow chart of extraction processes for the determination of OA in RAB done in 2003.**

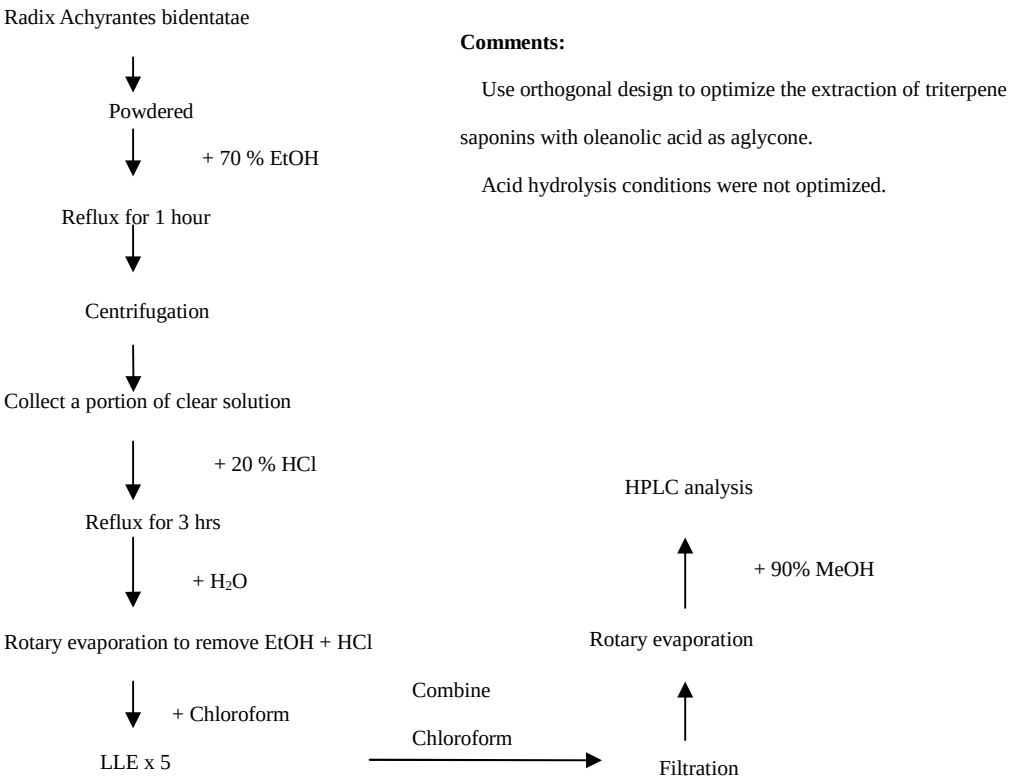


Figure 7 **Flow chart of extraction processes for the determination of OA in RAB done in 2005.**

Experiment

The aim of this experiment was to develop, optimize and validate a method for determination of OA in RAB, dried root of *Achyrantes bidentatae* Blume (Family *Amaranthaceae*). OA (marker of RAB) was prepared by complete acid hydrolysis of ethanolic extract of RAB and then measured quantitatively by using HPLC with DAD.

Optimization of extraction of OA from RAB

Method development

A simplified model from the previous research of OA extraction was described in flow chart (Figure 8). The powdered sample was undergone extraction, dilution, acid hydrolysis and then LLE prior to HPLC analysis.

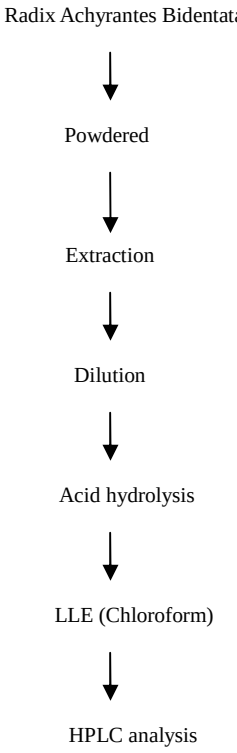


Figure 8 Simplified model from the previous research of OA extraction.

HPLC chromatographs for the extracts with hot extraction by 70% EtOH only, hot extracted by EtOH followed by acid hydrolysis and hot extracted by EtOH followed by acid hydrolysis and then extracted by LLE were described in figure 9. Comparable peak areas of OA in the chromatographs were obtained with and without LLE. Therefore, the steps of LLE can be omitted for the purpose of determining OA content.

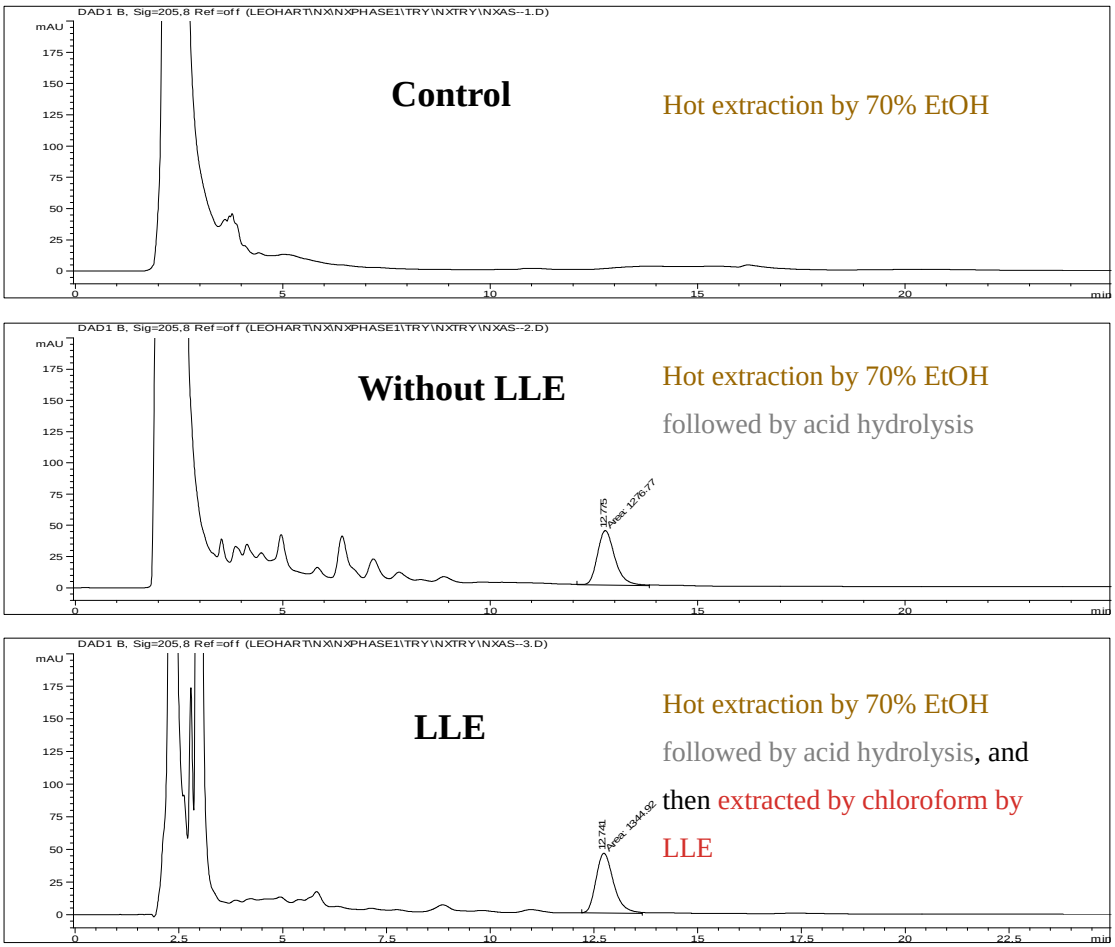


Figure 9 HPLC chromatographs for the extracts with hot extraction by 70% EtOH only, hot extracted by EtOH followed by acid hydrolysis and hot extracted by EtOH followed by acid hydrolysis and then extracted by LLE.

Extraction mechanism

Understanding extraction mechanism can help develop a more efficient extraction method. The powdered sample of RAB undergoes solvent extraction to extract the triterpene saponins. There are three saponins isolated from RAB by A Marouf in 2001 [7]. Acid hydrolysis is carried out to cleave off the sugar molecules to release OA as an aglycone. The major steps for determination of OA in RAB are solvent extraction and acid hydrolysis and described in figure 10. That was why RAB was chosen as the marker sample because the method developed required to determine the OA from saponins in addition to OA alone. So a simplified model was developed and described in figure 11. The powdered sample was undergone extraction, dilution and then acid hydrolysis prior to HPLC analysis. Dilution was used to lower the content of OA saponins and shorten the time of complete acid hydrolysis.

A method was developed according to extraction mechanism as described in figure 12. Powdered sample was hot extracted by 70 % EtOH for 1 hour and the extract was centrifuged. A potion of clear solution was heated under reflux with HCl for 3 hours. Then the mixture was rotary evaporated to remove the solvent. Finally the volume was made up with MeOH prior to HPLC analysis. Preliminary results of chromatograms of a blank, OA standard and sample were described in figure 13.

A solvent blank (MeOH), OA standard (1 mg/mL of OA in MeOH) and sample (0.2 g) were analyzed through the above steps. Results were positive (i.e. RAB contains OA) but the method could not be used for the determination because the conditions were not optimized. Further optimization was required for the determination.

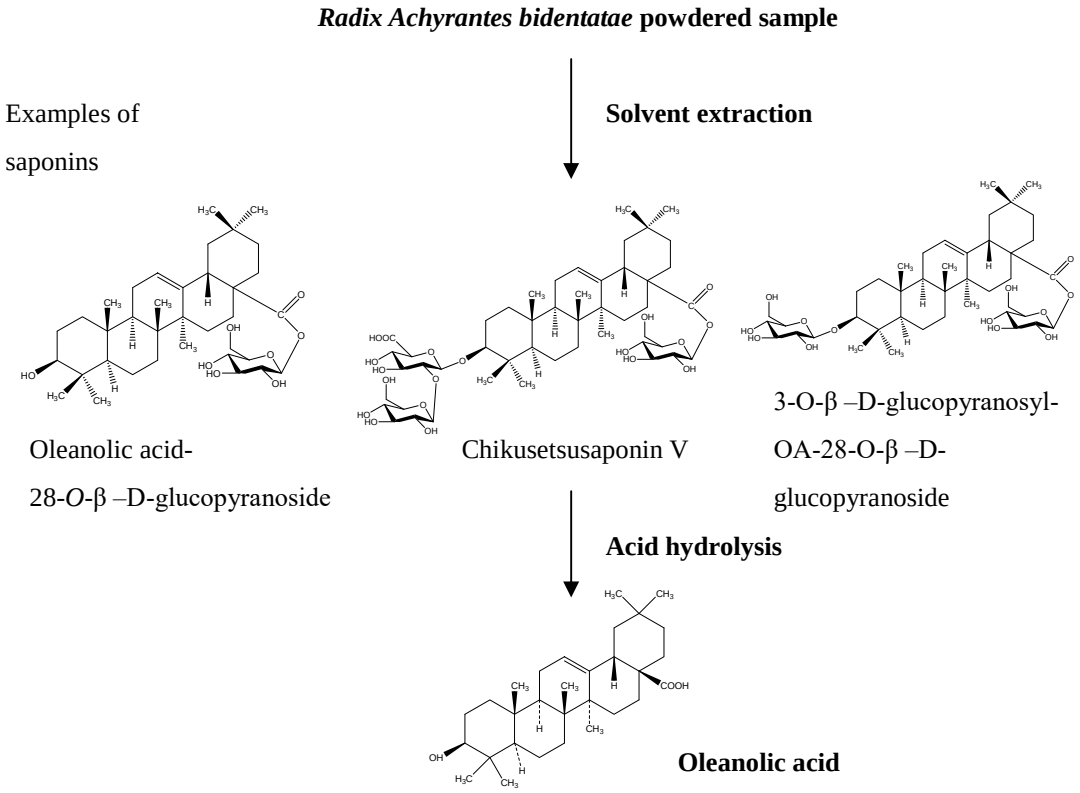


Figure 10 Mechanisms for extraction of OA from RAB.

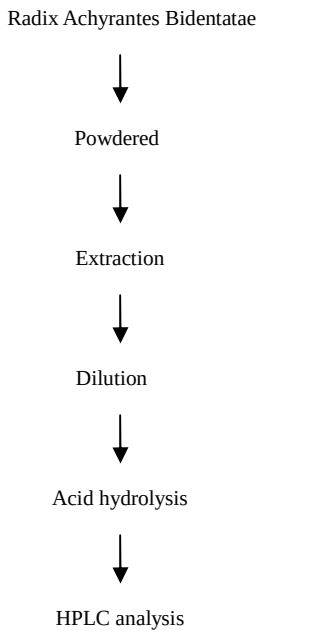


Figure 11 Simplified model for extraction of OA from RAB.

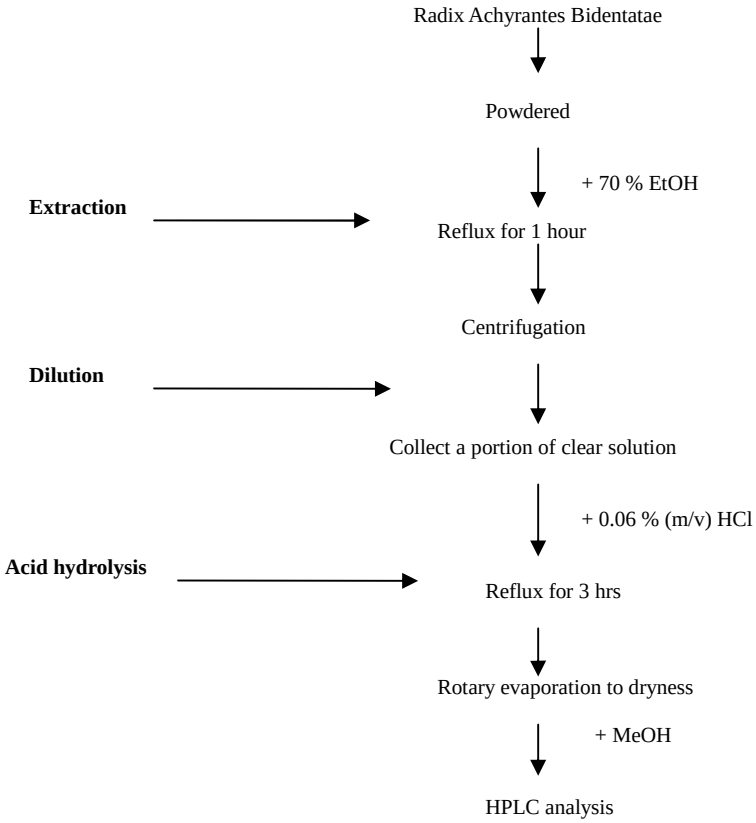


Figure 12 An unoptimized method for determination of OA from RAB.

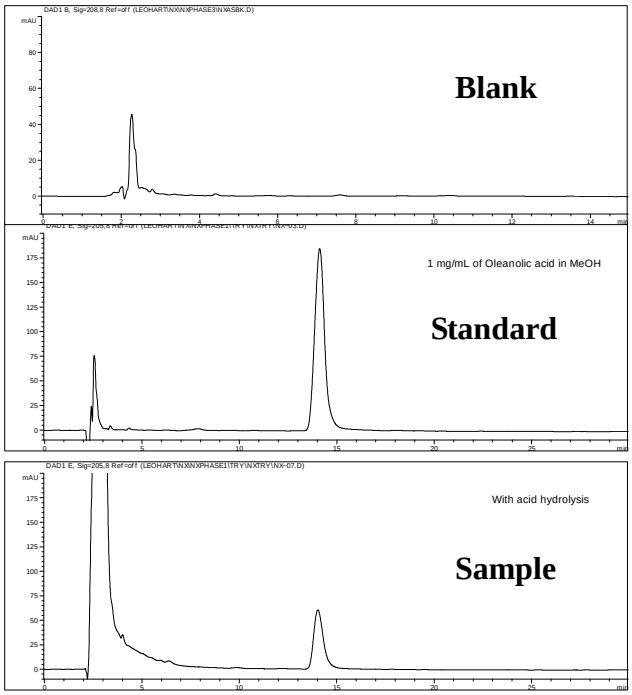


Figure 13 Chromatograms of a blank, OA standard and sample by an unoptimized method shown in figure 12.

Method optimization

Optimization is defined as to obtain a maximum stable result with minimum time and cost.

Extraction optimization

From the extraction steps of the previous un-optimized method (Figure 12), different concentration of EtOH and method of extraction were optimized (Figure 14). The first column shows different extraction procedures. Incubation represents incubate the powdered sample with 70% EtOH at 85 °C for 1 hour. Sonication represents sonicate the powdered sample with 70 % EtOH for 30 min. 3 times. The second column shows different dilution procedures. Filtered in V-flask means filter the solution into a 50 mL V-flask and then collect 10 mL of solution. Centrifugation means centrifuge the mixture and collect a portion of clear solution. The third column shows the procedures for acid hydrolysis. Incubation means Incubate with 0.06% (m/v) HCl at 75 °C for 22 hours. Reflux represents heat under reflux with 0.06% (m/v) HCl for 2 hours.
The last column shows the contents of OA extracted.

The procedures for conditions for dilution, acid hydrolysis and HPLC detection were remained unchanged for fair comparison. From the graph, 70 % EtOH with different extraction methods showed maximum results compared with 50% and 95% EtOH. Therefore, 70 % EtOH was used in the extraction procedures.

Optimization for different techniques for extraction, dilution and acid hydrolysis were needed to obtain a more desired result. The combination of different procedures of extraction, dilution and acid hydrolysis were described in figure 15. Compared with the procedures for acid hydrolysis, incubation had the yield significantly greater than reflux. So incubation was selected for acid hydrolysis instead of reflux. For dilution procedures, filtered in V-flask had the results greater than centrifugation, i.e. less loss for the procedures of filtered in V-flask. Compared with different extraction procedures, although incubation had an average result higher than sonication, repeatability was not as good as sonication. Finally, sonication was chosen due to the repeatable and comparable results with incubation. Therefore, sonication, filtered in V-flask and then incubation were used due to the maximum and repeatable results.

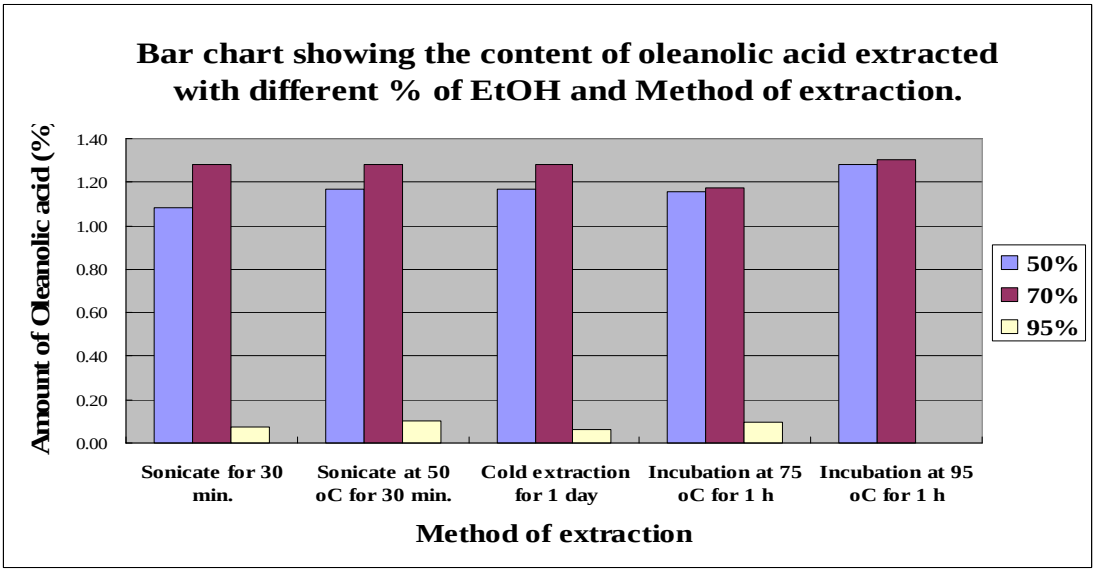


Figure 14 Bar chart showing the content of OA extracted with different % of EtOH and method of extraction. The conditions for dilution, acid hydrolysis and HPLC detection remained unchanged for fair comparison.

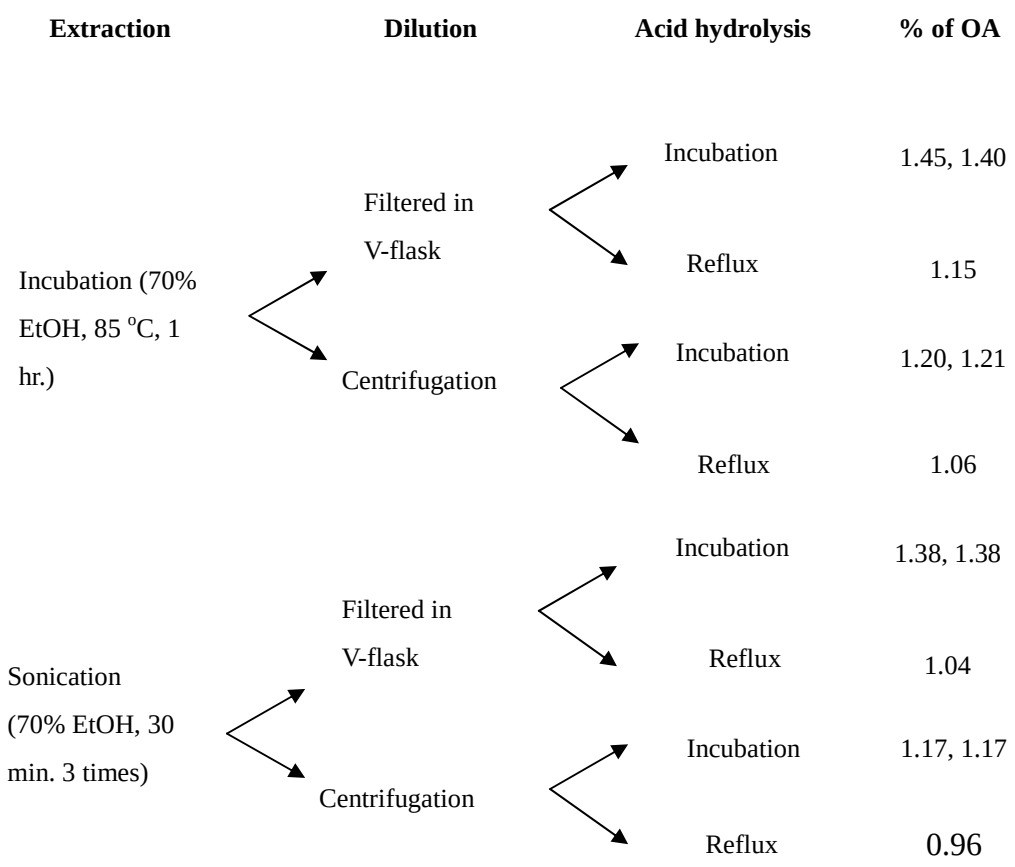


Figure 15 Flow chart showing the effect of combination of different procedures of extraction, dilution and acid hydrolysis on OA extraction from RAB.

Acid hydrolysis optimization

After the determination of extraction process, i.e. sonication, filtered in V-flask and then incubation, the next step is to optimize the time, acid concentration and temperature for acid hydrolysis. For temperature, 75 °C, 85 °C and 95 °C were tried.

For acid concentration, 0.03 %, 0.06 %, 0.09 % and 0.12 % HCl were used. For time, 1, 2, 3, 5, 7 and 28 hours were tried. The results were tabulated (Table 2). From table 2, the maximum content with minimum hydrolysis time was inspected as 1.5 % in which ethanolic extract was incubated at 85 °C for 7 hours with acid concentration of 0.06 % (m/v) HCl. However, compared with the result at the time of 28 hours, the condition 0.06 % HCl at 85 °C for 7 hours was 1.3 %. This means degradation of OA results by prolong hydrolysis. For the determination of method detection limit (MDL) in method validation, spiking 0.1 mg of OA in *bulbus fritillariae thunbergii* resulted in no OA signal due to the degradation of OA by prolong hydrolysis. As a result, using incubation condition with 0.06 % (m/v) HCl at 85 °C for 7 hours will result in a higher detection limit. The condition of incubation with 0.09 % (m/v) HCl at 95 °C for 1 hour was not used for the same reason.

The condition of incubation with 0.03 % (m/v) and 10% (m/v) HCl at 75 °C for 28 hours were searched as maximum and stable results. Prolong incubation times were analyzed until the completion of acid hydrolysis and the results were tabulated (Table 3). From the table, both the acid concentration of 0.03 % (m/v) and 0.06 % (m/v) HCl obtained the maximum value of 1.5 % in 28 hours. The condition of 0.06 % (m/v) HCl reached this value since 24 hours. This means using acid concentration of 0.06 % (m/v) HCl can obtain the maximum value with a shorter time. A

graph showing the percentage of OA in RAB extracted by acid hydrolysis with different incubation time was plotted (Figure 16).

From the curve, the formation of OA extraction was completed at about 22 hours at 75 ^{o}C when the acid conc. is 0.06 %.

Comparable result was obtained for heating under reflux at 75 ^{o}C with acid concentration of 0.06 % (m/v) HCl for 22 hours. However, simultaneous analyzing 24 samples at a time by incubation were more convenient than using one set of quick-fit apparatus for each sample by heating under reflux.

By inspection, the conditions used in acid hydrolysis done in previous research works were not optimized. i.e., acid hydrolysis was not completed.

Therefore, incubate the samples with 0.06 % (m/v) HCl at 75 ^{o}C for 22 hours was used in the acid hydrolysis procedures and a simplified flow chart of experimental procedures were described in figure 17. As a result, an optimized method for the determination of OA in RAB was developed and described in figure 18. The powdered sample was sonicated with 10 mL 70 % EtOH for 30 minutes 3 times and the mixture was filtered with regenerated cellulose membrane. The filtrate was combined and filled into a 50 mL V-flask and the volume was made up with 70% EtOH. Ten mL of clear solution in V-flask was incubated with 1 mL 6 N HCl at 75 ^{o}C for 22 hours. The solvent was removed by rotary evaporation and then 10 mL of MeOH was added to dissolve the solute. The mixture was filtered with regenerated cellulose membrane to a vial and HPLC analysis was carried out.

	Content of OA / %					
At 75 ^{o}C						
Acid Conc.\Time (hrs.)	**1**	**2**	**3**	**5**	**7**	**28**
0.03% (m/v) HCl	0.1	0.1	0.2	0.3	0.4	1.5
0.06% (m/v) HCl	0.1	0.4	0.5	0.7	0.8	1.5
0.09% (m/v) HCl	0.3	0.4	0.5	1.1	1.2	1.0
0.12% (m/v) HCl	0.4	0.8	0.9	1.1	1.4	1.1
At 85 ^{o}C						
Acid Conc.\Time (hrs.)	**1**	**2**	**3**	**5**	**7**	**28**
0.03% (m/v) HCl	0.1	0.4	0.7	1	1.1	1.3
0.06% (m/v) HCl	0.5	0.7	1	1.3	1.5	1.3
0.09% (m/v) HCl	0.6	0.9	1.2	1.1	1.1	1.2
0.12% (m/v) HCl	0.8	1.2	1.4	1.3	1.3	1.1
At 95 ^{o}C						
Acid Conc.\Time (hrs.)	**1**	**2**	**3**	**5**	**7**	**28**
0.03% (m/v) HCl	0.6	0.9	1.1	1.1	1.1	0.8
0.06% (m/v) HCl	0.7	1	1.2	0.9	1.1	0.8
0.09% (m/v) HCl	1.5	1	1.1	0.7	0.6	0.6
0.12% (m/v) HCl	1.2	1.3	1	0.9	1.2	0.7

Table 2 Percentage of OA in RAB when using different temperature, acid concentration and time for acid hydrolysis.

	Content of OA / %														
Incubation time (hrs.) / acid conc.	**1**	**2**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**	**21**	**23**	**24**	**28**
0.03% (m/v) HCl	0.1	0.1	0.2	0.3	0.4	0.5	0.6	0.8	0.9	0.9	1.1	1.0	1.1	-	1.5
0.06% (m/v) HCl	0.1	0.4	0.5	0.7	0.8	0.9	1.1	1.2	1.2	1.3	1.4	1.4	1.4	1.5	1.5

Table 3 **Percentage of OA of RAB extracted with prolong incubation times until the completion of acid hydrolysis.**

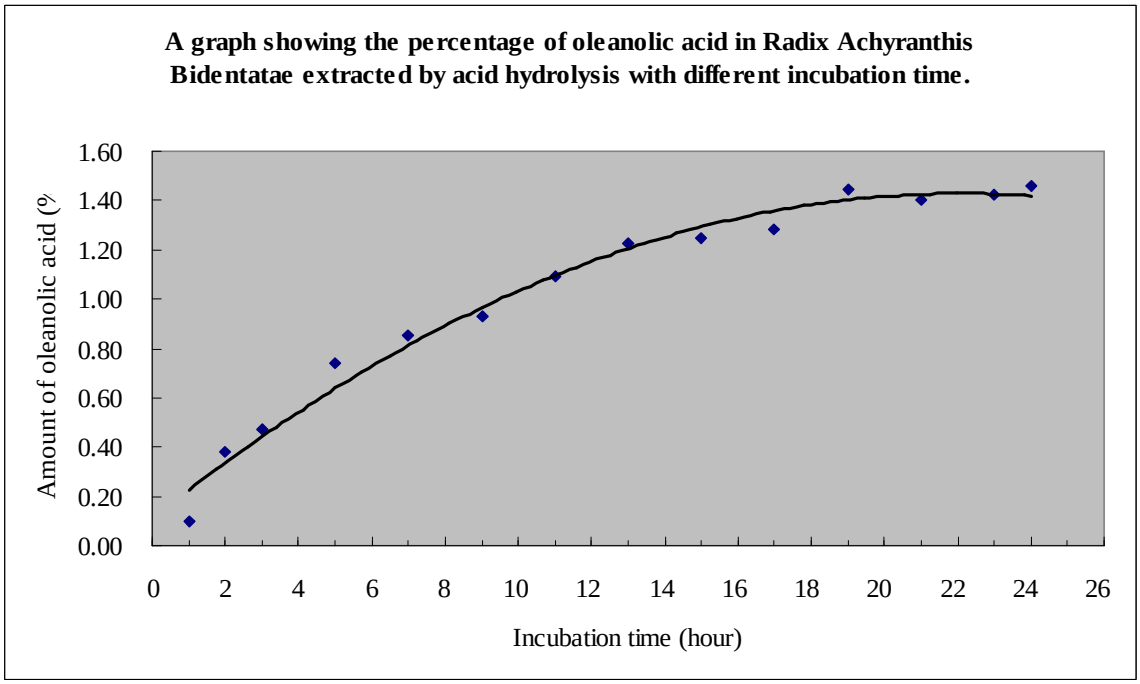


Figure 16 **A graph showing the percentage of OA in RAB extracted by acid hydrolysis with different incubation time.**

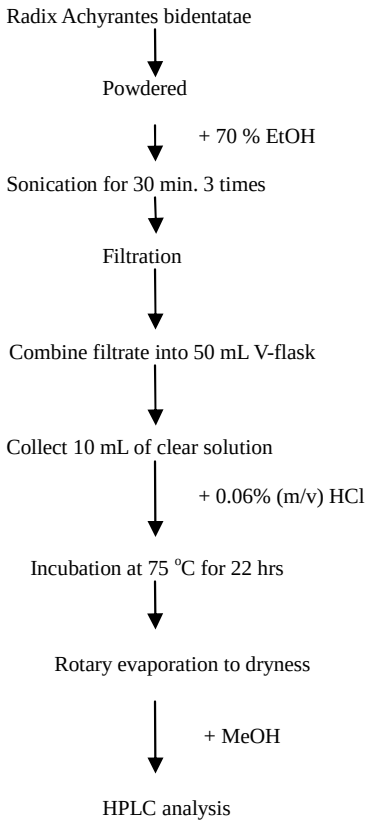


Figure 17 Flow chart of the method of extraction after optimizing extraction procedures and conditions of acid hydrolysis.

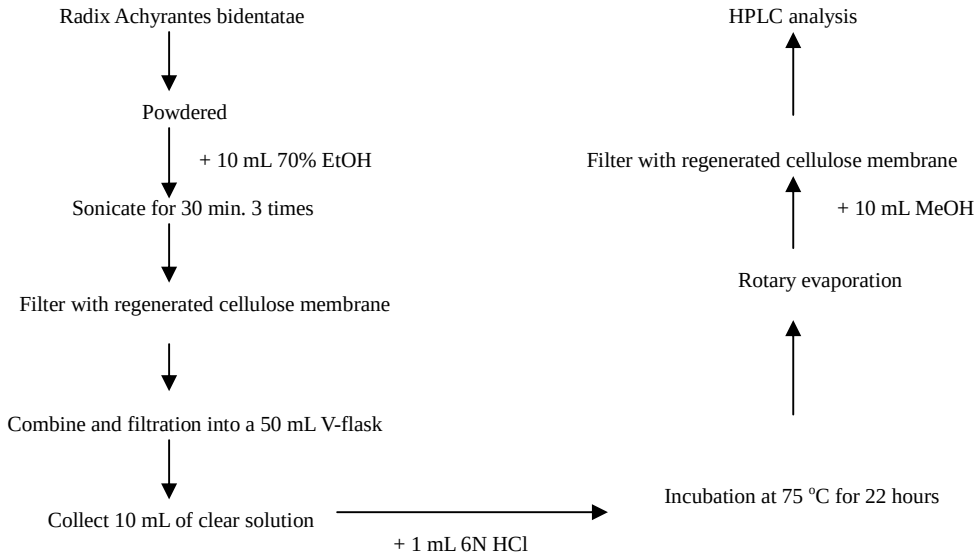


Figure 18 Flow chart of the procedures of extraction after optimizing extraction procedures and conditions of acid hydrolysis.

Optimization of HPLC separation and detection

From the OA ultraviolet (UV) absorption spectrum (Figure 19), OA has UV absorption range from 190 - 225 nm. It has maximum absorption at 208 nm. So 208 nm was chosen as the detection wavelength.

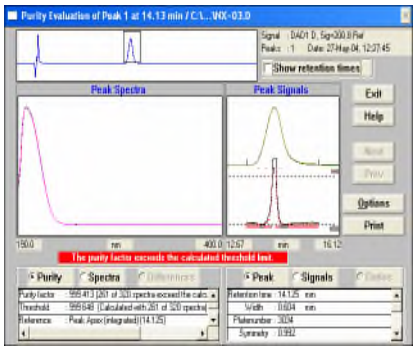


Figure 19 OA UV absorption spectrum

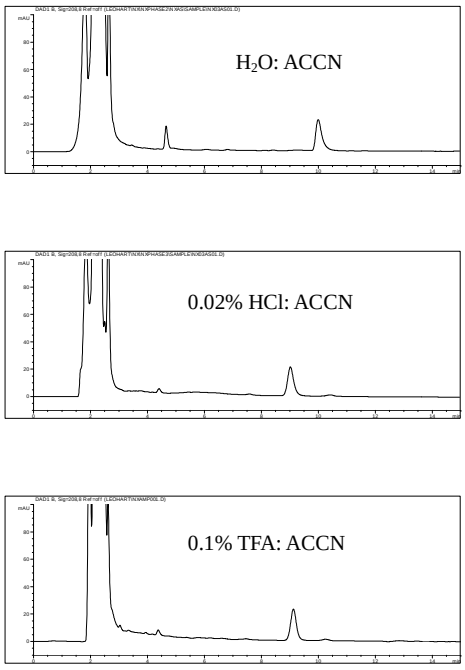
From the Van Deemter plot [10-12], flow rate of HPLC does not change so much on the height equivalent to theoretical plate. This means flow rate is not the key factor affecting resolution. Therefore, flow rate of 1.0 mL/min was chosen as the detection flow rate.

MeOH was not used as the mobile phase because of its absorption at detection wavelength of 208 nm. Solvent system: H_2O: ACCN, 0.02 % HCl: ACCN and 0.1 % Trifluroacetic acid (TFA): ACCN were tried (Figure 20). Peak tailing was shown in water: ACCN system. Comparable results were obtained for 0.02 % HCl: ACCN and 0.1 % TFA: ACCN solvent system.

Therefore, 0.02 % HCl: ACCN was chosen because TFA has UV absorption at wavelength 208 nm. It would be difficult to convert to gradient elution if isocratic elution cannot separate OA from sample interference.

Isocratic elution was tried before gradient elution (Figure 21). Peak tailing were shown when using 0.02 % HCl: ACCN of 0:100 and 10:90 solvent system. The 0.02 % HCl: ACCN of 30:70 solvent systems were not used because of the broadening of peak results in lower sensitivity. As a result, 0.02 % HCl: ACCN of 20:80 was chosen

The HPLC conditions used were tabulated (Table 4). The optimized HPLC conditions were determined to be running isocratic elution of 0.02 % HCl: Acetonitrile (2:8) at the flow rate of 1 mL/min with the UV detection wavelength of 208 nm.



Peak Tailing

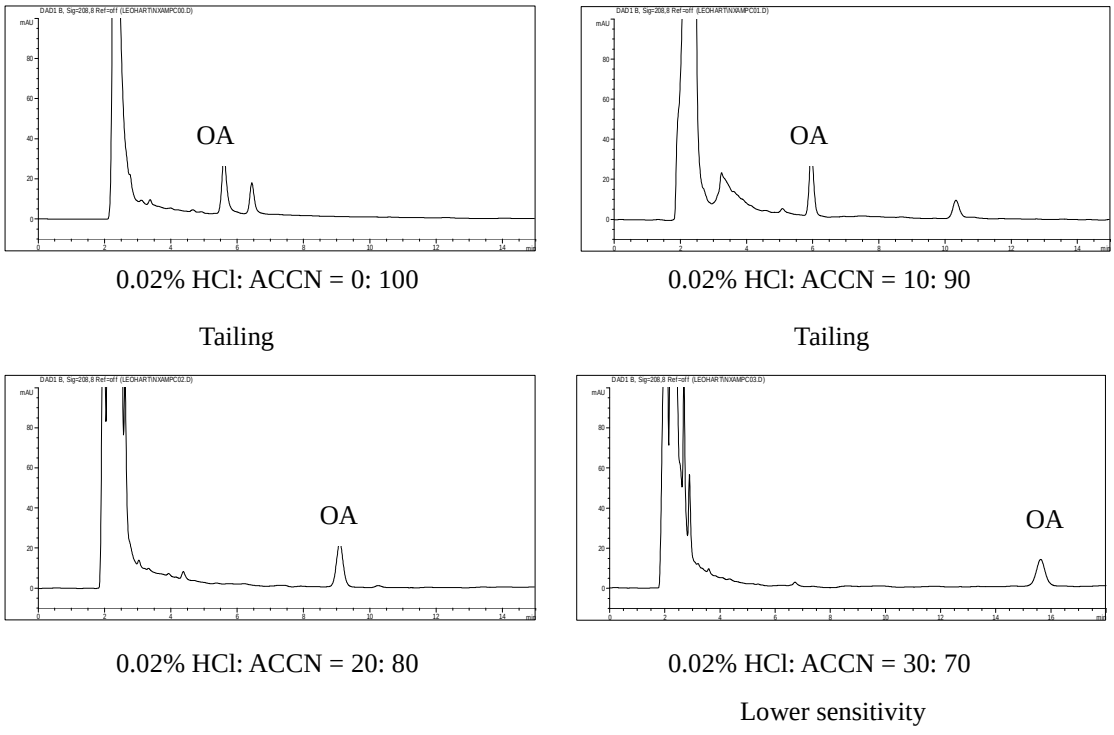
Comparable to 0.02% HCl: ACCN but difficult to convert to gradient elution at wavelength at 208 nm (if needed to convert)

Figure 20 Chromatographs showing optimization of solvent system: H_2O: ACCN, 0.02% HCl: ACCN and 0.1% TFA: ACCN for detection of OA.

Factors of HPLC	Conditions
Instrument	Agilent 1100 series
Column	Waters Nova-Pak C_{18} HPLC c 300 mm, 4 μm or equivalent
Mobile phase	0.02% HCl : ACCN (2:8, v/v)
Injection volume	10 μL
Detector	DAD
Detector wavelength	208 nm
Flow rate	1.0 mL/min
Run time	15 min

Table 4 Optimized conditions of HPLC used for determination of OA from RAB

– Isocratic elution



0.02% HCl: ACCN = 0: 100

Tailing

0.02% HCl: ACCN = 10: 90

Tailing

0.02% HCl: ACCN = 20: 80

0.02% HCl: ACCN = 30: 70

Lower sensitivity

Figure 21 Chromatographs showing optimization of composition of solvent system (0.02% HCl: ACCN)

Method validation

Reagents and apparatus

Reagents used shall be of chromatographic reagent grade or equivalent unless otherwise specified. MeOH, ACCN and 6N HCl were HPLC grade, EtOH was AR-grade. Water was deionized. OA was obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) of China. The purity of markers was tested by three different wavelengths (205 nm, 208 nm, 218 nm). The percentage purity was set to be the one with the lowest percentage purity (Table 5). i.e. 96.9 %. EtOH stock solution (70% EtOH): Seven hundreds mL of EtOH was added into 300 mL of water. OA stock standard solution: About 4.0 mg of OA was weighed accurately and dissolved in 4 mL of MeOH.

HPLC system equipped with the following components: LC pump capable of gradient flow rate up to 1.0 mL/min and with two solvent inlets, injector with a 10-µL sample loop, DAD or UV detector with detection wavelength of 208 nm, C_{18} HPLC column, 3.9 x 300 mm, 4 µm or equivalent; Regenerated cellulose, Syringe filter, 4 mm x 0.45 µm; Syringe, 1mL and 20 mL; Centrifugal tube, 50 mL; Analytical balance, readable to 0.1 mg; Centrifuge, with nominal speed of *ca.* 12000 rpm or other speed where necessary; Ultrasonic bath; Blender or mill; Sieve, No. 2 conforming to Pharmacopoeia of the People's Republic of China 2005, Volume 1 or equivalent; Autopipettes, 0.2 mL, 1 mL and 5 mL; Measuring cylinder, 50 mL; Water bath, 75 °C, shaking at ca. 40 rpm; V-flask, 10 mL and 50 mL.

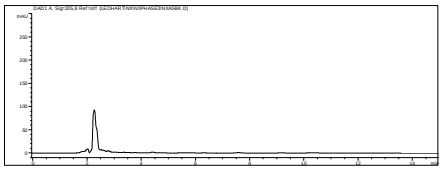
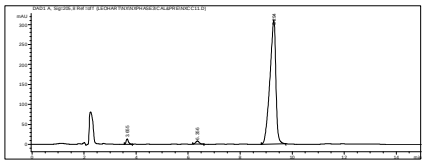
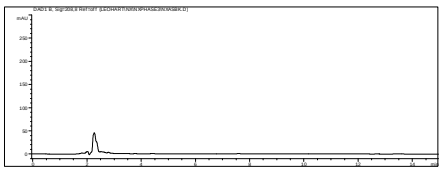
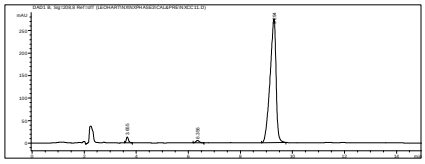
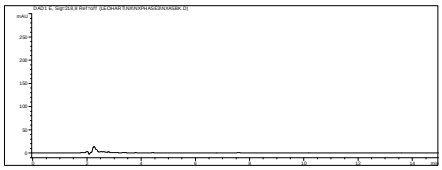
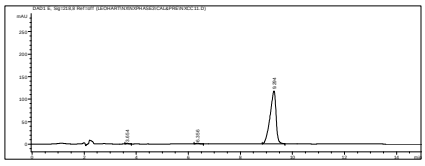
Detection wavelengths / nm	Chromatograms of Blank	Chromatograms of OA
205		
208		
218		

Table 5(a) Chromatograms of a solvent blank and OA at different wavelenths.

Wavelength (nm)	205	208	218
Impurity peak area (mAU*s)	81.8	81.1	9.2
	77.4	49.4	14.6
Marker peak area (mAU*s)	4953.1	4380.3	1871.5
Sum of peak area (mAU*s)	5112.3	4510.8	1895.3
Percentage purity (%)	96.9	97.1	98.7

Table 5(b) Table showing the impurity peak area, marker peak area and percentage purity of OA.

Experimental Procedure

Sampling

Sample was powdered before analysis. Grind at least 5 times the quantity of sample to be used for analysis or the entire sample, whichever is the less.The powder was passed through a No. 2 sieve.

Extraction

About 0.2 g of sample was accurately weighed into a 50-mL centrifugal tube. Ten mL of 70 % ethanol was accurately added into the tube. The mixture was sonicated for 30 minutes with occasional shaking. The tube was mixed and centrifuged at ca. 12000 rpm (or other speed where necessary) for 8 minutes. The solution was filtered with regenerated cellulose filter membrane. The procedures of adding 10 mL 70 % ethanol to filtering solution with regenerated cellulose filter membrane were repeated twice.

Dilution

The filtrate was combined into a 50-mL V-flask and the V-flask was filled up to the graduated mark with 70 % ethanol. Ten mL of mixture was mixed and pipetted into a 50-mL centrifugal tube.

Acid hydrolysis

One mL of 6N HCl was pipetted into the mixture. The mixture was shaken in a 75 ^{o}C water bath for 22 hours. The mixture was allowed to cool to room temperature. The solution was transferred into a 50-mL round bottomed flask. The residue liquid was rinsed into the round bottomed flask by 5 mL of 70 % EtOH for three times. The solvent was evaporated to dryness using a rotary evaporator. Three mL of MeOH was added to the tube to dissolve the residue. The solution was transferred into a 10 mL V-flask. The tube was rinsed with 3 mL of methanol for 2 times. The V-flask was filled to the mark with methanol. The solution was filtered with regenerated cellulose filter membrane. The filtrate was ready for HPLC analysis.

The steps of extraction, dilution and acid hydrolysis was repeated for a method blank, a sample duplicate and a spiked sample (0.1 g sample + 1.5 mg OA or any amount of standards deemed appropriate).

This experiment is focus on the determination of OA content, and not the extraction of pure OA. Purifying OA from the acid hydrolysate from subsequent CC and/or crystallization will cause the loss of contents of OA due to human error in repeated column chromatography and re-crystallization, underestimating OA content.

HPLC/UV Analysis

HPLC system was set up according to the manufacturer's manual. HPLC conditions for analysis were set up according to Table 4.

The entire system was equilibriated for 20-30 minutes. Calibration curves were established by injecting 10 µL each of the working standard solution into the HPLC system for analysis. Under the recommended LC conditions as shown, the retention time of OA is about 9.0 minute. HPLC analysis for the method blank, sample, sample duplicate and spiked sample solutions was performed. Appropriate dilution was made if the concentrations of marker in samples fall outside the calibration range.

Identification of OA was based on comparing the retention time (RT) of OA in sample with that of the nearest checking standard solution. The retention time of the marker obtained in sample and standard should agree within 5 %. The content of OA was reported to 3 significant figures. HPLC/UV chromatograms of RAB for the determination of OA were shown in figure 22-26.

Quality Control Parameters

System suitability test

Developed by Martin and Synge, a chromatographic column is described in terms of a distillation column or a liquid extractor. (Figure 27)

Separation efficiency = concentration of A_1 / concentration of A_2.

A chromatographic column is envisaged as being composed of a series of discrete but continuous, narrow, horizontal layers called theoretical plates. At each plate, equilibration of the solute between the mobile phase and the stationary phase is assumed to take place (Figure 28).

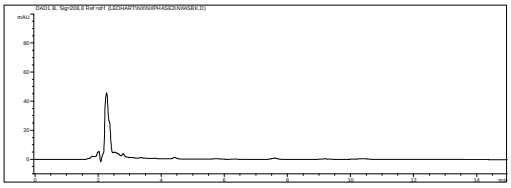


Figure 22 HPLC/UV Chromatogram of a method blank for the determination of OA

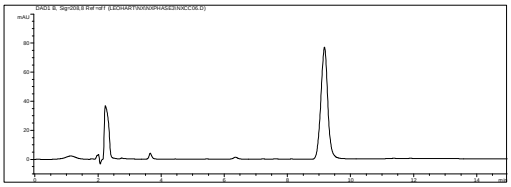


Figure 23 HPLC/UV Chromatogram of a standard solution (0.2 mg/mL) for the determination of OA

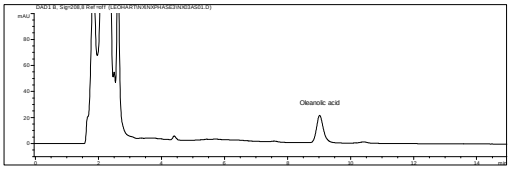


Figure 24 HPLC/UV Chromatogram of 0.2 g RAB (Peak area of 317.5 mAu) for the determination of OA

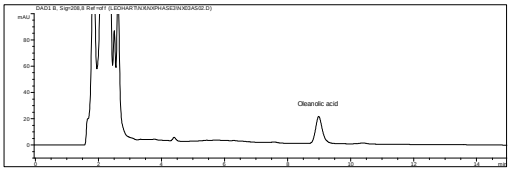


Figure 25 HPLC/UV Chromatogram of 0.2 g RAB duplicate for the determination of OA

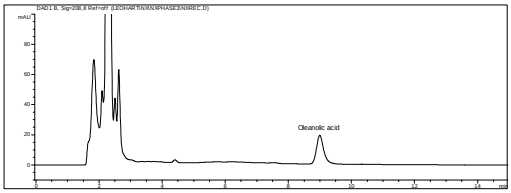


Figure 26 HPLC/UV Chromatogram of 0.1 g RAB spiked with OA (peak area of 304.2 mAu) for the determination of OA

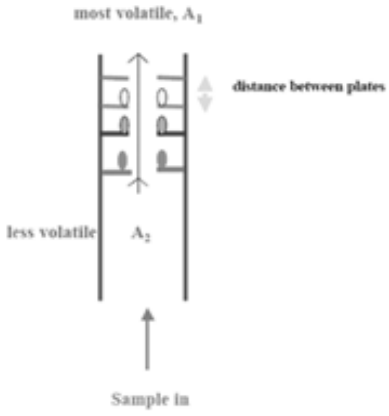
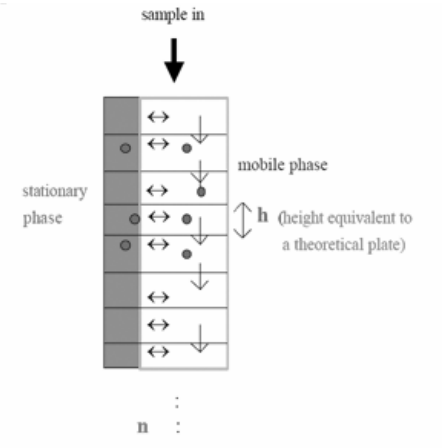


Figure 27 Chromatographic column developed by Martin and Synge



n = theoretical plate number

h = height equivalent to a theoretical plate

Figure 28 A chromatographic column envisaged as being composed of a series of discrete but continuous, narrow, horizontal layers called theoretical plates

Movement of the solute and mobile phase is viewed as a series of stepwise transfers from one plate to the next. The efficiency of a chromatographic column as a separation device improves as the number of equilibrations increases i.e. theoretical plate number also increases.

Thus, efficiency increases, theoretical plate number increases or height equivalent to a theoretical plate decreases

As the solute band passes through a chromatographic column, it broadens and the resultant profile of the solute band closely approaches that given by a Gaussian distribution curve (Figure 29).

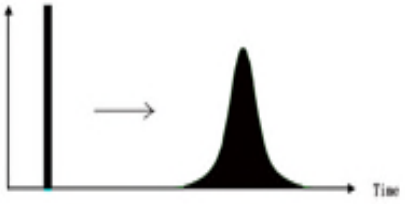


Figure 29 Resultant profile of the solute band broadens and closely approaches to give a Gaussian distribution curve as solute band passes through a chromatographic column.

From the profile of an absolute band (Figure 30(a)), the theoretical plate number is defined as $(RT / \sigma)^2$, as $W = 4\sigma$ or $\sigma = W/4$, theoretical plate number = $16 (RT/W)^2$. As $\sigma = W_{1/2} / (8 \ln 2)^{1/2}$, theoretical plate number = $5.54 (RT/W_{1/2})^2$.

In the measurement of theoretical plate number, uncorrected retention time is used which can overestimate the efficiency. Thus, the effective plate number takes into account i.e., from figure 30(b), Effective theoretical plate number = $16 (RT_R - RT_M / W)^2 = 16 (RT_R / W)^2$, and effective theoretical plate number = $5.54 (RT_R / W_{1/2})^2$, whereas RT_M = retention time of an unretained solute, RT_R = retention time of the solute

The resolution, R, between any two compounds is described as:

$R = 2\Delta t / (W_{b1} + W_{b2})$ (Figure 31).

$R \geq 1$ (satisfactory resolution, 96 % resolution) (Figure 32)

$R \geq 1.5$ (baseline, 99.7 % resolution) (Figure 33)

Tailing factor (TF) = $W_{0.05h} / 2d_1$ (Figure 34).

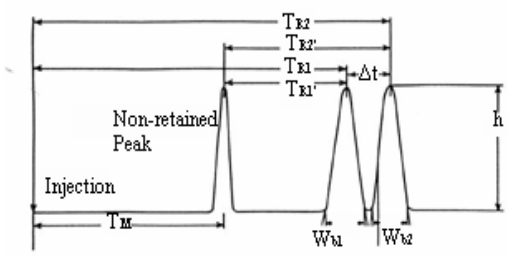
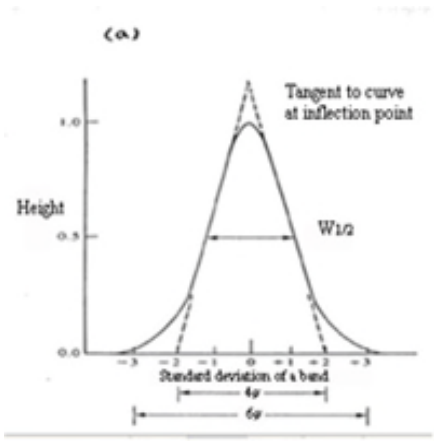


Figure 31 Chromatographic profiles of peaks for calculating resolution R



RT = Retention time of the solute

σ = standard deviation of the solute band

W = band width of the solute band

$W_{1/2}$ = width at half of the peak height.

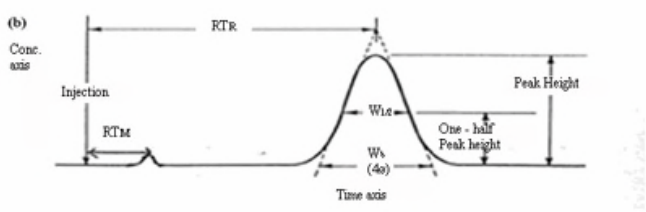


Figure 30 (a) Profile of a solute band (b) evaluation of a chromatographic band for column efficiency.

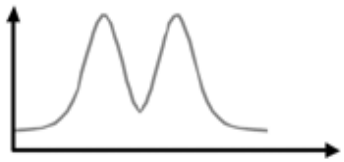


Figure 32 Chromatographic profiles of peaks with satisfactory resolution

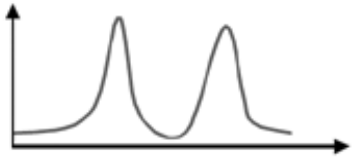


Figure 33 Chromatographic profiles of peaks with baseline resolution

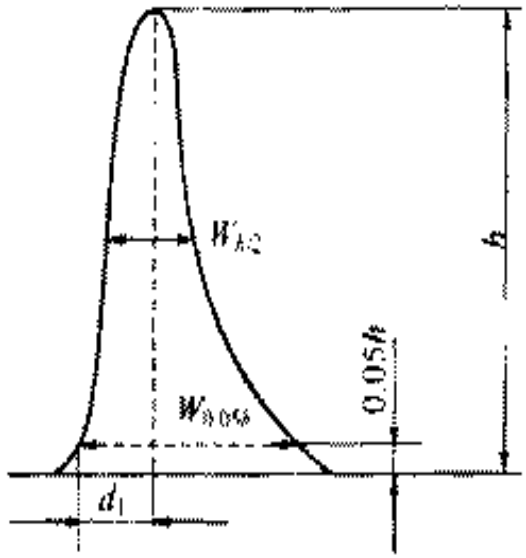


Figure 34 Chromatographic profile of peak for calculating tailing factor.

TF should be within 0.95 to 1.05. Value out of this range will affect the accuracy for the determination of small peak.

Resolution (R), number of theoretical plate (N) and tailing factor (TF) of OA peak in HPLC profile of RAB were tabulated (Table 6).

Sample	Marker	RT (min)	$W_{h/2}$ (min)	N	R	TF
NX 03	OA	9.020	0.2244	8948	2.94	1.221
NX 04	OA	9.005	0.2311	8410	3.07	1.214
NX 08	OA	8.953	0.2333	8157	2.91	1.228

Table 6 Table showing the retention time (RT), width with half height ($W_{h/2}$), number of theorectical plate (N), resolution (R) and tailing factor (TF) of peak of OA in HPLC profiles of threes sources of RAB samples.

The retention time (RT), width with half height ($W_{h/2}$), number of theorectical plate (N), resolution (R) and tailing factor (TF) of peak of OA in HPLC profiles of threes sources of RAB samples were tabulated (Table 6). From the table, N ≥ 6000 and R of the marker with the closest peak ≥ 1.5 which gives baseline separation. TF are within 1.221 to 1.228 which is out of range within 0.95 to 1.05. So the concentration/dilution step in the extraction procedures is necessary to adjust in order to avoid small peak in the chromatogram. Fortunately, the content of OA in RAB is high enough to gain sensitivity and dilution steps are required to avoid peak overloading. If the peak is small, concentration steps are required to compensate tailing effect.

Reproducibility

Reproducibility of peak area (PA) and retention time (RT) for five replicates analysis of OA from RAB were tabulated (Table 7).

Trial	PA (mAU*s) of OA	RT of OA (min)
1	321.71985	9.02
2	312.2399	8.99
3	337.2471	9.045
4	337.586	9.025
5	331.633	8.944
Mean	328.09	9.005
RSD (%)	3.3	0.44

Table 7 Reproducibility of peak area (PA) and retention time (RT) of five replicate analysis of OA from RAB.

Area reproducibility of OA is < 5% relative standard deviation (RSD) and retention time reproducibility of OA < 2% RSD. As a result, the method has high reproducibility of PA and RT. i.e. high accuracy.

Detection Limit

Method detection limit (MDL) is the concentration or amount in extract needed to produce a signal five times the signal to noise ratio in which the samples containing the constituent of interest are processed through the complete analytical method. Seven portions of the solution over a period of 3 days were prepared and analyzed. Standard deviation (SD) was calculated and MDL was computed. Replication measurements should be in the range of one to five times the calculated MDL. From the table of one-sided t distribution select the value of t for 7-1 = 6 degrees of freedom and at the 99 % level of 3.14. MDL of a constituent of interest is the analyte concentration producing a signal of at least 3.14 times the standard deviation of 7 determinations of a CHM sample.

MDL of determining OA from RAB was determined by spiking *bulbus fritillariae thunbergii* with the marker and the results were tabulated (Table 8).

Trial	OA (mg/L)
1	0.130
2	0.096
3	0.118
4	0.145
5	0.097
6	0.134
7	0.140
Mean (mg/L)	0.123
SD (mg/L)	0.020
RSD (%)	16.18
MDL in Solution (mg/L)	0.06
MDL in Sample (mg/kg)	15.6
Spiked amount (mg)	0.005
Spiked Conc. in Sample (mg/kg)	25
Spiked Conc. in Solution (mg/L)	0.1
Mean Recovery (%)	123.01

Table 8 OA content for seven replicates analysis by spiking *bulbus fritillariae thunbergii* with OA which produce a signal five times the signal to noise ratio through the complete analytical method of determinating OA from RAB. Standard deviation (SD) was calculated and MDL was computed. Replication measurements should be in the range of one to five times the calculated MDL. From the table of one-sided t distribution select the value of t for 7-1 = 6 degrees of freedom and at the 99% level of 3.14. MDL of a constituent of interest is the analyte concentration producing a signal of at least 3.14 times the standard deviation of 7 determinations of a CHM sample.

The calibration curve used for determinating MDL of OA from RAB was shown in figure 36. HPLC chromatogram of *bulbus fritillariae thunbergii* without spiking OA and have a background peak similar to OA was shown in figure 37 and HPLC chromatogram of *bulbus fritillariae thunbergii* spiked with OA at 15.6 mg/kg was shown in figure 38.

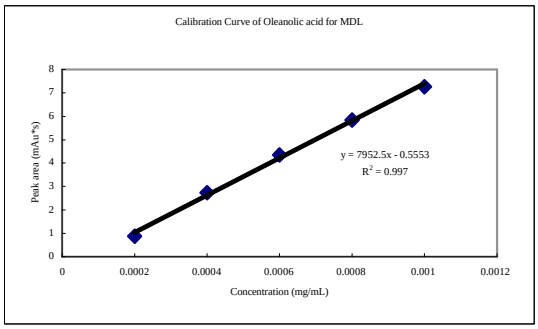


Figure 36 Calibration curve of OA for MDL

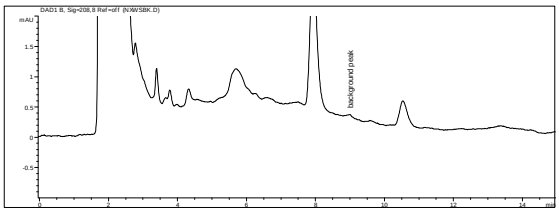


Figure 37 HPLC chromatogram of *bulbus fritillariae thunbergii* without spiking OA and have a background peak similar to OA.

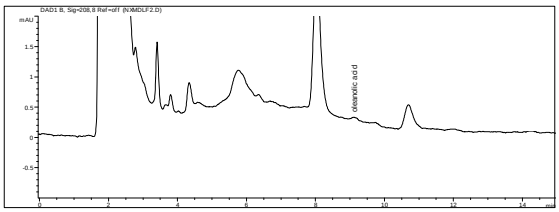


Figure 38 HPLC chromatogram of *bulbus fritillariae thunbergii* spiked with OA at 15.6 mg/kg.

As a result, MDL of the method for determination of OA in RAB in sample and testing solution were determined to be 15.6 mg/kg and 0.06 mg/L respectively with the spiking amount of 0.005 mg.

Limit of quantitation (LOQ)

Limit of quantitation (LOQ) has been proposed as the lowest level achievable among laboratories within specified limits during routine laboratory operations. LOQ is significant because different laboratories will produce different MDLs even though using the same analytical procedures, instruments, and sample matrices. LOQ is about

five times the MDL and represents a practical and routinely achievable detection level with a relatively good certainty that any reported value is reliable.

LOQ was determined by spiking *Bulbus fritillariae thunbergii* with 15.6 µg of OA which was 5 times the MDL, i.e. 78.15 mg/kg for OA for a sample size of 0.2 g with a final make-up volume of 10 mL. The results and calculations were tabulated (Table 9). HPLC chromatogram was shown in figure 39.

Trial	OA (mg/L)
1	0.412
2	0.436
3	0.429
4	0.455
5	0.429
Mean Conc.	0.432
Spiked Amount (µg)	15.6
Amount Found (µg)	15.5
SD	0.02
RSD (%)	3.58
Mean Recovery (%)	99.1

Table 9 Five replicates analysis of OA by spiking Bulbus fritillariae thunbergii with 15.6 µg of OA. i.e. 5 times the MDL = 78.15 mg/kg of OA for a sample size of 0.2 g with a final make-up volume of 10 mL for determination of LOQ.

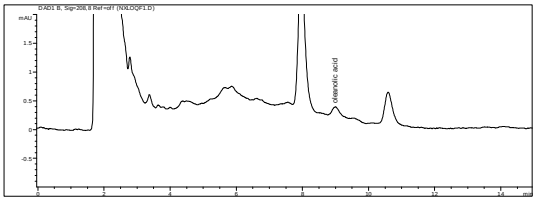


Figure 39 HPLC chromatogram of *bulbus fritillariae thunbergii* spiked with OA at 78.15 mg/kg.

The mean recovery of OA was calculated as 99.1% which were quantitative.

LOQ was set at 5 times the MDL. It was set at 78.15 mg/kg for OA for a sample size of 0.2 g with a make-up volume of 10 mL.

Linearity study

Concentrations of 1, 5, 10, 50, 100, 200, 400, 500, 600, 800 mg/L of OA were prepared for HPLC analysis. A linearity curve of OA was plotted (figure 40).

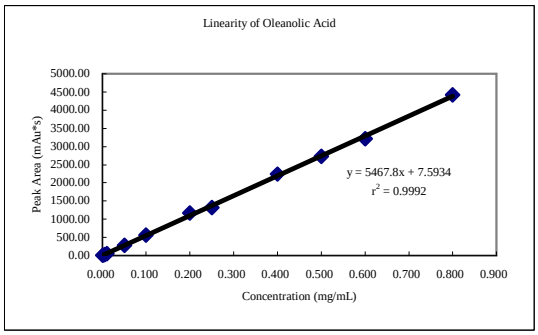


Figure 40 Calibration curve for linearity study of OA

The correlation coefficient (r^2) was determined to be 0.9992 which was > 0.995. A good linearity was resulted.

Calibration curve

Concentration of OA 1, 10, 50, 100, 400 mg/L were prepared. Dilution table for calibration curve of OA was prepared (Table 10).

Table 10 Dilution table for calibration curve of OA

Standard code	Volume of standard solution	Volume of MeOH (µL)	*ca.* conc. of OA (mg/L)
Std5	400 µL of standard stock solution	600	400
Std4	100 µL of standard stock solution	900	100
Std3	500 µL of Std4	500	50
Std2	200 µL of Std3	800	10
Std1	100 µL of Std2	900	1

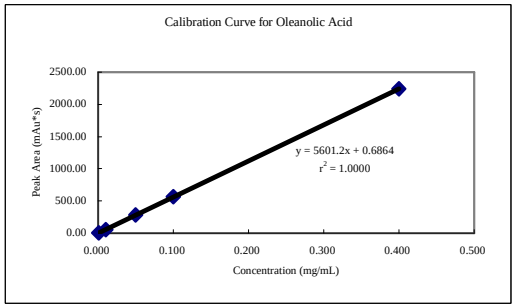


Figure 41 Calibration curve of OA

Calibration curve of OA was plotted (Figure 41). Correlation coefficient (r^2) was calculated as 1.0000, which was greater than 0.995. As a result, calibration curve had good linearity for quantification.

Concentration of OA in RAB was calculated according to the following equation:

(Concentration of OA in the sample solution determined from the calibration curve of the respective standard OA solution) x (Final make-up volume of testing solution) x (dilution factor) x (volume factor (50/10) x [(peak area in the sample solution - y-intercept of the calibration curve) / (slope of the calibration curve)] / (Sample weight)

Precision

Five replicate injections of 50.0 mg/L standard solution of OA were carried out. The mean concentration found and RSD were calculated and tabulated (Table 11).

Trial	OA (mg/L)
1	49.80
2	50.17
3	50.20
4	50.18
5	50.21
Mean (mg/L)	50.11
SD (mg/L)	0.18
RSD (%)	0.35
Standard conc. (mg/L)	50.00

Table 11 Precision of 5 replicate injections of OA standard solutions and RSD was calculated.

The mean concentration found was determined to be 50.11 mg/L and RSD was determined to be 0.35%. High precision was resulted.

Repeatability

Repeatability study is the average means and RSDs for 5 replicate analyses of test samples. Five replicate analyses of RAB extracts were carried out for OA determination. Mean and RSD were calculated and tabulated (Table 12).

Trial	OA (mg/kg)
1	14257
2	13892
3	14910
4	14925
5	14683
Mean (mg/kg)	14533
SD (mg/kg)	448.7
RSD (%)	3.09

Table 12 Repeatability of 5 replicate analysis of RAB for determination of OA.

Mean concentration was determinated to be 14533 mg/kg and RSD was determined to be 3.09 %.

Recovery

Recovery study is the average recoveries and RSDs for 5 replicate analyses of test samples spiked with marker. Five replicate analyses of half of weight of RAB spiked with half of mean content of OA from RAB were carried out. Mean recovery and RSD were calculated and tabulated (Table 13).

Table 13 Recovery of 5 replicate analysis of RAB for determination of OA. Mean recovery and RSD were calculated.

Trial	OA recovery (%)
1	96.83
2	95.21
3	96.55
4	96.64
5	97.08
Mean (%)	96.46
SD (%)	0.73
RSD (%)	0.76
Spiked amount (mg)	3.0

The spike amount was calculated as 3.0 mg. Mean recovery and RSD were determined to be 92.14 % and 1.02% respectively. The mean recovery was within 90 - 100 % acceptance limit.

Experimental results

Duplicate analysis of OA determination for 10 different sources of RAB was carried out. The results were tabulated (Table 14).

Batch No	Sample code	OA content (mg/kg)	OA content (%)	OA content Mean (%)
1	LNXS 001	13539.40	1.35	1.38
1	LNXS 001(d)	14028.91	1.40	
2	LNXS 003	14131.54	1.41	1.46
2	LNXS 003(d)	15138.92	1.51	
3	LNXS 004	17816.24	1.78	1.80
3	LNXS 004(d)	18247.10	1.82	
4	LNXS 005	17369.90	1.74	1.72
4	LNXS 005(d)	17049.00	1.70	
5	NXS 003	16437.37	1.64	1.62
5	NXS 003(d)	15910.34	1.59	
6	NXS 004	15831.90	1.58	1.60
6	NXS 004(d)	16144.92	1.61	
7	NXS 005	12234.43	1.22	1.22
7	NXS 005(d)	12244.85	1.22	
8	NXS 006	14019.15	1.40	1.37
8	NXS 006(d)	13346.70	1.33	
9	NXS 007	14777.71	1.48	1.49
9	NXS 007(d)	14976.25	1.50	
10	NXS 008	15635.73	1.56	1.62
10	NXS 008(d)	16812.54	1.68	
		15284.65	1.53	1.53
		1766.87	0.18	0.18
		11.6	11.6	11.6

Table 14 Results of duplicate analysis of determination of OA from RAB.

The mean contents and RSD of RAB from duplicate analysis of OA for 10 different sources were determined to be 1.53% and 11.6% respectively.

Determination of OA from natural products

A developed, optimized and validated method for determination of OA from RAB was described in figure 2.40 and the optimized HPLC conditions were tabulated (Table 15).

Factors of HPLC	Conditions
Instrument	Agilent 1100 series
Column	Waters Nova-Pak C_{18} HPLC column, 3.9 x 300 mm, 4 µm or equivalent
Mobile phase	0.02% HCl : Acetonitrile (2:8, v/v)
Injection volume	10 µL
Detector	DAD
Detector wavelength	208 nm
Flow rate	1.0 mL/min
Run time	15 min

Table 15 Optimized HPLC conditions for determination of OA from RAB.

The optimized procedures were mainly divided into four parts: extraction, dilution, acid hydrolysis and HPLC analysis. Chromatograms of a blank, OA standard solution, sample, a sample duplicate and a sample spiked with OA were shown in figure 42.

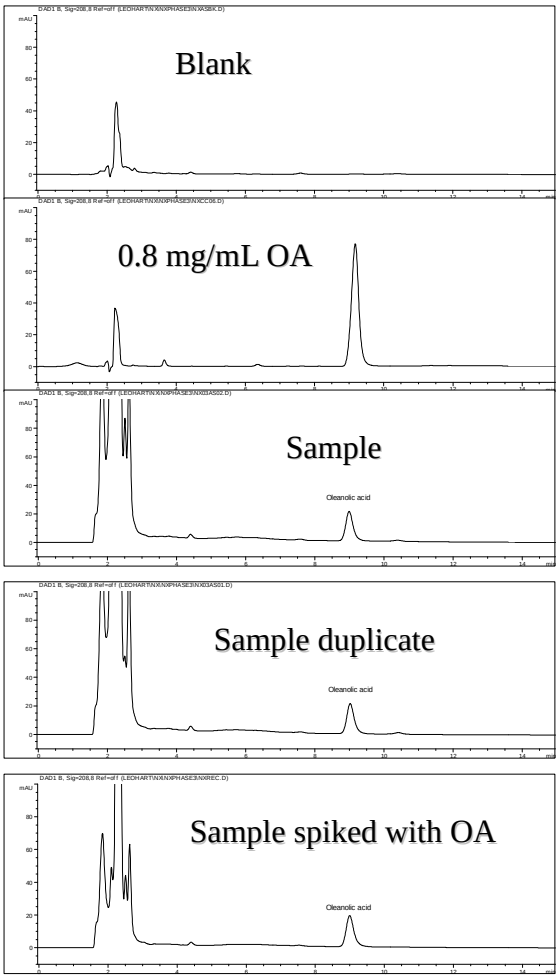


Figure 42 Chromatographs of a blank, OA standard solution, sample, a sample duplicate and a sample spiked with OA.

To determine OA in other medicinal plant, four major optimization steps were needed. They are:

Optimization of the number of times of 70 % EtOH extraction (N_{noe})

Number of times of extraction was required to be optimized to ensure complete extraction of OA and triterpene saponins.

Concentration or dilution

Concentrating and diluting extracts was required to maximize peak sensitivity and prevent overloading of column respectively during HPLC analysis.

Optimization of time of acid hydrolysis (T_{ah})

Time of acid hydrolysis was needed to be optimized to ensure complete hydrolysis of triterpene saponins to OA. The temperature and acid concentration for acid hydrolysis were optimized as 75 ^{o}C and 0.06 % (m/v) HCl respectively previously in which the conditions had nearly no degradation of OA.

Optimization of HPLC conditions

The step of acid hydrolysis was included in the extraction procedures. A lot of components were breaking down into small and polar substances which elute earlier in the HPLC profile. As shown in the chromatogram of determination of OA in RAB, a lot of overlapping peak at the beginning of the chromatogram and a clear peak of OA with RT of about 9 minutes. HPLC conditions were needed to be re-optimized again if impurities appeared on the chromatogram.

Therefore, the method of OA determination for natural products was developed and the flow chart of extraction procedures was described in figure 43. The powdered natural products was sonicated with 70 % EtOH for 30 minutes N_{noe} times, in which the number of times of extraction was needed to be optimized. The mixture was then filtered with regenerated cellulose membrane. The filtrate was combined into a suitable V-flask or rotary evaporated to dryness and then known amount of 70 % EtOH was added, depends on dilution or concentration needed.

Ten mL of clear solution was incubated with 1 mL 6N HCl or final acid concentration of 0.06 % (m/v) HCl at 75 ^{o}C for T_{ah} hours was made, in which the time of acid hydrolysis was needed to be optimized. The solvent was removed with rotary evaporation and then 10 mL MeOH was added to dissolve the solute. The mixture was filtered with regenerated cellulose membrane before HPLC analysis. HPLC conditions were needed to be optimized if there was overlapping peaks in the HPLC profile. This developed method not only determines OA content present in natural products, but also OA exists in a form of aglycone in triterpene saponins in natural products.

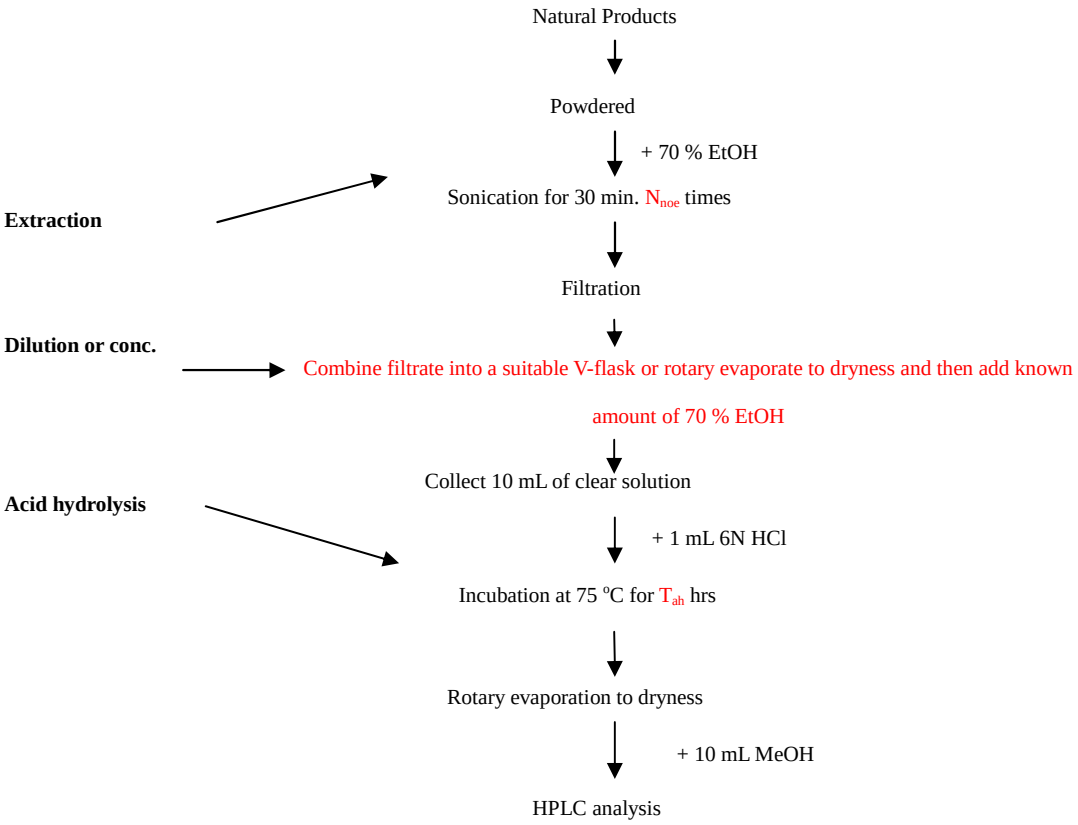


Figure 43 Flow chart of method developed for determination of OA from natural products.

Table 17 Extraction methods and HPLC conditions for determination of oleanolic acid from different plant materials used by other laboratories.

Plant materials	Extraction methods	HPLC conditions	Content/%	RSD/%	References
-*Swertia mussotii*	Extraction ($CHCl_3$, and	Supelco INC C_{18}	0.480	1.17	[13]
-*Swertia franchetiana*	then 50% EtOH, and then	column (150 nm x 1.5	0.289	1.21	
-*Swertia peyewalskiij*	75% EtOH)	mm x 5 μm)	0.175	0.98	
-*Swertia erythrusticta*	Reflux (2 hrs., n=3)	M. p. (ACCN : H_2O =	0.365	1.27	
-*Swertia tetraptera*	Filtration.	85:15)	0.018	1.34	
-*Gentianopsis paludosa*	Dissolvation (MeOH).	λ = 215 nm	0.130	1.28	
-*Halenia elliptica*			0.159	1.21	
-*Lomatogonium rotatum*			0.173	1.16	
-*Gentiana ferreri*			0.172	1.19	
-*Ziziphus zizyphus* (Linn.) H..	Extraction (petroleum	C_{18} column (Type	0.1153	1.23	[14]
Karst.	ether, 10 min.)	Waters, 4.6 x 150 mm)	0.1385	1.54	
	Sonication (10 min.).	M.p. (ACCN: MeOH:	0.1021	1.39	
	Filtration.	H_2O = 70:16:14)			

	Drying of residue. Soxlet extraction (ether, 2 hrs.) Vacuum evaporation. Dissolvation (MeOH)	λ = 215 nm R = 0.8 mL/min			
-*Ziziphus zizyphus* (Linn.) H.. Karst.	Sonication (75% MeOH, 5 min., n=3) Filtration Rotary evaporation Dissolvation (MeOH)	Hydersir BDS C_{18} column (200 x 4.6 mm, 5μm) M.p. (MeOH : H_2O = 90:10, KH_2PO_4, pH 3) λ = 210 nm R = 0.6 mL/min	0.0220 0.0205 0.0177 0.0251 0.0170 0.0231 0.0084 0.0153 0.0125 0.0102 0.0102 0.0100 0.0085	3.39	[15]
Wild jujube			0.0379		
-*Herba Plantaginis* -*Calyx. Kaki* -*Herba Oldenlandia Diffusae* -*Herba Cynomorii* -*Fructus Ligustri Lucidi* -*Fructus Gardeniae* -*Fructus Ligustri Lucidi* -*Fructus Chaenomelis* -*Fructus Crataegi* -*Fructus Mume* -*Percarpium granati*	Sonication (95% EtOH, 30 min., n=3) Filtration Rotary evaporation. Dissolvation (MeOH)	ODS C_{18} column (250 mm x 4.6 mm, 5 μm) M.p. (MeOH : H_2O : CH_3COOH : $(C_2H_5)_3N$ = 90:10:0.03:0.06), λ = 210 nm R = 0.6 mL/min	0.1212 0.1132 0.2251 0.0880 0.8028 0.0252 0.2513 0.0038 0.1364 0.0407 0.0203	2.36	[16]
-mung bean Honghu Xiaogan -red bean Honghu Wuhan -lotus seed Honghu	Reflux (EtOH, 1 hr., n=3) Filtration Rotary evaporation Acid hydrolysis (reflux, 20 mL 4 mol/L HCl, 30 min., 100 °C) LLE ($CHCl_3$, 10 mL, 15 min., n=3)	Nucleodur C_{18} column (250 mm x 4.6 mm, 5 μm) M.p. (EtOH : H_2O = 80:20), λ = 210 nm R = 1.0 mL/min	0.0141 0.0121 0.0177 0.0191 0.0016	2.05 2.17 1.59 1.09 2.79	[17]

Dongxihu	Rotary evaporation		0.0011	2.77	
-jujube	Dissolvation (80% EtOH)				
Shenlongjia			0.0010	2.89	
Hebei			0.0089	1.07	
-*Fructus crataegi*	Optimization of conc. and volume of EtOH, and time of cold extraction. The conc. of EtOH has sig. impact and the volume and time of extraction have less impact on the extraction.	C_{18} column (250 mm x 4.6 mm, 10 μm) M.p. (MeOH : H_2O = 90:10), R = 0.5 mL/min	-	-	[18]
-*Herb Lycopi* -*Folium Eriobotryae* -*Spica Prunellae* -*Radix Ilicis Pubescentis* -*Herba Artemisiae Scopariae* -*Fructus Ligustri Lucidi* -*Caulis Perillae* -*Fructus Crataegi* -*Fructus Forsythiae* -*Herba Hedyotidis Diffusae*	Acid hydrolysis (reflux, 28 mL EtOH, 12 mL 6 mol/L HCl, 2 hrs,) Neutralization (NaOH, to pH 5) Dissolvation (EtOH) Centrifugation	Shim-pack CLC-ODS column (150 mm x 6 mm, 5μm) M.p. (MeOH : H_2O : HOAc : TEA = 83:17:0.04:0.02) λ = 210 nm R = 1.0 mL/min	0.076 0.203 0.058 0.021 - 0.780 0.094 0.059 0.499 0.110	1.8	[19]
-*Fructus Ligustri Lucidi* -*Spica Prunellae* -*Herba Verbenae* -*Fructus Corni* -*Fructus Crataegi* -*Fructus Chaenomelis* -*Liuwei Dihuang* pills -*Zhibai Dihuang* pills -*Mugua* pills -*Erzhi* pills -*Dashanzha* pills in Beijing -*Dashanzha* pills in chifeng	Sonication (MeOH, 40 min.)	Kromasil C_{18} column (4.6 mm x 150 mm, 5μm) M.p. (MeOH : H_2O : HOAc = 260:40:0.15) λ = 204 nm R = 0.6 mL/min	0.910 0.120 0.150 0.080 0.360 0.310 0.014 0.012 0.020 0.140 0.026 0.019	-	[20]
-*Ligustrum lucidum*	Optimization of conc. of EtOH, time and temperature of extraction by orthogonal design	-	-	-	[21]

	Sonication (75% EtOH, 40 ^{o}C, 45 min.) Precipitation ($Pb(Ac)_2$) Centrifugation Collect liquid Rotary evaporation Dissolvation (MeOH)				
- *Fructus Ligustrum lucidum*	Comparison of sonication and soxlet extraction. Comparison of the no. of hours of extraction. Sonication (MeOH, 30 min., n=1)	Shim-Pack C_{18} column M.p. (ACCN : MeOH: H_2O: NH4Ac = 70:16:14:0.5) λ = 215 nm R = 1.0 mL/min	0.561 0.608 0.764 0.749		[22]
-*Anoectochilus roxburghii*	Supercritical fluid extraction (55^{o}C, 25 MPa, Supercritical fluid CO_2, modifier EtOH, H_2O and EtOAC, 1 hour static, 1 hour dynamic) Soxlet extraction (EtOH, 3 hours, n=3) Centrifugation Filtered Rotary evaporation Freeze drying (24 hours) Dissolvation (MeOH)	HPLC-MS Agilent Zorbax C_{18} column (250 mm x 4.6 mm x 5 μm) Temp = 30 ^{o}C M.p. (Acetic acid : H_2O : MeOH = 1:15:84) R = 0.8 mL/min. λ = 210 nm	1.18	3.12	[23]
--*Fructus Ligustri lucidi* Crude drug Braised product Alcohol processed product Vinegar processed product	Sonication (MeOH, 20 min., n=3)	Shim-Pack-ODS column (151 mm x 4.6 mm, 5 μm) M.p. (MeOH : H_2O = 95:5) λ = 215 nm R = 1.0 mL/min.	1.658 1.947 2.227 1.862	2.29 1.54 0.45 1.14	[24]
--*Fructus Ligustri lucidi* Jiejiang Xichuan Meinan Shanxi	Sonication (MeOH, 30 min.)	Lichrospher C_{18} column (4.6 mm x 250 mm, 5 μm) M.p. (MeOH : 0.5%	1.04 1.12 0.83	1.7	[25]

Baoji, Shanxi		CH_3COONH_4 = 90:10)	0.90		
Nanning, Guangxi			0.99		
Jiangsu			1.32		
Fujian			1.89		
Hunan			0.78		
Henan			1.53		
Hefei, Anhui			0.65		
-*Fructus Ligustri lucidi*	Reflux (95% EtOH 40	-	1.10		[26]
	min., n=4)		0.93		
	Add H_2O and HCl to pH 1.		1.07		
	Neutralization (NaOH)		0.98		
	Boiling (20 min.)		0.94		
	Precipitation				
	Dissolvation (EtOH)				
	Boiling				
	Crystallization				
-*A. echinocaulis*	Soxlet extraction (MeOH,	Shim-pack LC-ODS			[27]
Root	3 hrs.)	column (6.0 mm x 150			
2002-03-20	Acid hydrolysis (reflux,	mm, 5 μm)	1.86	0.02	
2002-04-15	20% H_2SO_4, 4 hrs.)	M.p. (MeOH : H_2O =	1.77	0.12	
2002-04-30	LLE ($CHCl_3$, n=5)	90:10)	1.69	0.08	
2002-05-15	Rotary evaporation	λ = 220 nm	1.66	0.04	
2002-05-31	Dissolvation (MeOH)	R = 1.2 mL/min.	1.67	0.09	
2002-06-15			1.48	0.03	
2002-06-30			1.46	0.03	
2002-07-15			1.48	0.05	
2002-07-31			1.50	0.02	
2002-08-15			1.88	0.11	
2002-08-31			2.28	0.07	
2002-09-15			2.43	0.14	
2002-09-30			2.48	0.07	
2002-10-15			1.93	0.08	
Stem					
2002-03-20			1.43	0.05	
2002-04-15			1.38	0.07	
2002-04-30			1.38	0.02	
2002-05-15			1.32	0.04	
2002-05-31			1.45	0.03	

2002-06-15			1.45	0.09	
2002-06-30			1.42	0.10	
2002-07-15			1.36	0.05	
2002-07-31			1.33	0.07	
2002-08-15			1.39	0.12	
2002-08-31			1.41	0.11	
2002-09-15			1.40	0.05	
2002-09-30			1.42	0.08	
2002-10-15			1.42	0.02	
Leaves					
2002-03-20			1.01	0.02	
2002-04-15			0.56	0.01	
2002-04-30			0.58	0.03	
2002-05-15			0.61	0.02	
2002-05-31			0.65	0.04	
2002-06-15			0.69	0.01	
2002-06-30			0.72	0.03	
2002-07-15			0.73	0.03	
2002-07-31			0.75	0.05	
2002-08-15			0.77	0.02	
2002-08-31			0.78	0.02	
2002-09-15			0.80	0.04	
2002-09-30			0.81	0.06	
2002-10-15			0.81	0.02	
-Aralia taibaiensis	Optimization of acid hydrolysis by orthogonal design (powdered size, fertilization of powder, time and acid conc. for hydrolysis) Reflux (70% EtOH n=3, 1 hr.) Dilution Acid hydrolysis (reflux, 7% H_3PO_4, 4 hrs.)	Hypersil ODS C_{18} column (4.6 mm x 150 mm, 10 μm) M.p. (MeOH : H_2O: H_3PO_4 = 90:10:0.006) λ = 220 nm R = 1.0 mL/min.	4.71	-	[28]

	Neutralization (NaOH, HCl) Soxlet extraction ($CHCl_3$) Dilution Dissolvation (MeOH)				
-*Aralia taibaiensis*	Soxlet extraction (MeOH, 3 hrs.) Acid hydrolysis (reflux, 10% H_2SO_4, 4 hrs.) LLE ($CHCl_3$, n=5)	Shim-pack LC-ODS (6.0 mm x 150 mm, 5 μm) M.p. (MeOH : H_2O = 90:10) λ = 220 nm R = 1.2 mL/min.	0.91 1.79 0.94 1.10 2.97 1.65	0.01 0.03 0.05 0.03 0.02 0.02	[29]
-*Fructus Forsythiae*	Sonication (95% EtOH, 120 min., n=3)	Nova-Pak C_{18} column (3.9 mm x 300 mm, 4 μm) M.p. (MeOH : H_2O = 88:12) λ = 210 nm R = 0.8 mL/min.	0.328 0.322 0.348 0.404	1.8 1.9 2.0 2.2	[30]
-*Chaenomeles speciosa*	Optimization by orthogonal design (EtOH Conc., solvent volume, time and temp. of extraction) Soxlet extraction (95% EtOH, 1:10 solute: solvent ratio, 2 hrs., 60 °C) LLE ($CHCl_3$, n=3) Rotary evaporation Dissolvation (95% EtOH)	-	2.86	4.36	[31]
-*Chaenomeles Lagenaria*	Cold extraction (95% EtOH) Filtration Rotary evaporation C.C. (petroleum ether and then ether)	-	0.982	-	[32]

	Rotary evaporation of ether fraction. Crystallization (95% EtOH)				
-*Papaya*	Extraction (95% EtOH, 60 ^{o}C, 2 hrs.) Repeat to decolorization of extracts.	Kromasil C_{18} column (250 mm x 4.6 mm, 5 µm) M.p. (MeOH : CH_3COOH = 100:0.1) λ = 215 nm R = 1.0 mL/min.	0.0601	2.45	[33]
-*Swertia erythrosticta Maxim.* -*S. franchetiana H. Smith* -*Gentiana farrer Balf. f.* -*S. tetraptera Maxim.* -*S. przewalskii Pissjauk* -*S. mussotii Franch.* -*Gentianopsis pacedosa (manro) Ma.* -*Halenia ellipitica D. Don* -*Lomatogonium rotatum (L.) Fries ex Nym.*	Sonication (MeOH, 40 min., n=2)	Kromasil C_{18} column (250 mm x 4.6 mm, 5 µm) M.p. (MeOH : 0.04% H_3PO_4 = 87:13) λ = 215 nm R = 0.8 mL/min.	0.0370 0.1207 0.3086 0.1315 0.1730 0.0910 0.2674 0.2466 0.3304	0.72-0.90	[34]
-*Hemsleya Chinensis Cogn.*	Optimization by orthogonal design (Pressure, temperature and time of extraction, vol. of entrainer) Comparison on SFE-CO_2, sonication and reflux. SFE-CO_2 extraction (40 MPa, 55 ^{o}C, EtOH, 2 hrs., flow of CO_2 = 20 kg/h)	ODS Hypersil C_{18} column (250 mm x 4.6 mm, 5 µm) M.p. (MeOH : ACCN : 0.1% CH_3COONH_3 = 18:68:14) λ = 215 nm R = 1.0 mL/min.	0.31		[35]
-*Chaenomeles lagenaria*	Reflux ($CHCl_3$, 1 hr., n=3)	ODS C_{18} column M.p. (ACCN : 0.1% H_3PO_4 = 80:20)	0.0831 0.0792		[36]

		λ = 210 nm			
		R = 1.0 mL/min.			
-*Fructus Corni*	Soxlet extraction (ether, 4	Shim-Pack C_{18} column			[37]
Jiangsu	hrs.)	(5 μm, 150 mm x 4.6	0.0624	2.2	
Jiangsu	Cold extraction (petroleum	mm)	0.0728		
Anhui	ether, 30-60 ^{o}C, 2 min.,	M.p. (ACCN : MeOH :	0.0612		
	n=2)	H_2O : CH_3COONH_3 =			
-*Zhi Bai Di Huang* Wan	Rotary evaporation	70:16:14:0.5)	0.0059		
	Dissolvation (MeOH)	λ = 215 nm	0.0063		
		R = 1.0 mL/min	0.0054		
-*Plantago asiatica* L.	Extraction (95% EtOH, 3	C_{18} ODS column	3.54	1.66	[38]
-*Plantago depressa* Willd.	min.)	M.p. (MeOH : H_2O =	3.22	1.56	
	Suction filtration	88:12)			
	Rotary evaporation	λ = 220 nm			
	Soxlet extraction (ether, 3	R = 0.6 mL/min			
	hrs.)				
	Rotary evaporation				
	Dissolvation (MeOH)				
-*Clematis manshurica*	Reflux (H_2O, 1 hr.)	Kromasil C_{18} column	0.2281	1.52	[39]
-*Clematis chinensis*	Suction filtration	(4.6 mm x 150 mm, 5	0.1260		
-*Clematis hexapetala*	Optimization of time and	μm)	0.1851		
	acid conc. of acid	M.p. (MeOH : H_2O =			
	hydrolysis	95:5)			
	Acid hydrolysis (reflux, 10	λ = 220 nm			
	% HCl, n=3)	R= 0.5 mL/min			
	LLE (EtOAc, n=3)				
	Rotary evaporation				
	Dissolvation (MeOH)				
-*Hedyotis corymbosa*	Reflux (EtOH, 2 hrs.)	Hypersil ODS C_{18} (150			[40]
Wuhan company		mm x 4.6 mm, 5 μm)	0.075	2.3	
Huangpo, Wuhan		M.p. (MeOH : 0.1%	0.081		
Xinzhou Wuhan		H_3PO_4 = 82:18)	0.069		
		λ = 210 nm			
-*Hedyotis diffusa* Willd.		R = 1.0 mL/min			
Wuhan company			0.016		
Huangpo, Wuhan			0.016		
Xinzhou, Wuhan			0.015		

-*Aralia chinensis*	Extraction (MeOH, n=3)	Shimpack (LC-ODS 6,0	0.7332	0.035	[41]
-*Aralia taibaiensis*	Suction filtration	mm x 150 mm, 5 μm)	1.3261	0.013	
-*Aralia dasyphylla*	Rotary evaporation	λ = 220 nm	0.2828	0.064	
-*Aralia echinocaulis*	Acid hydrolysis (Reflux,	R = 2 mL/min	0.0394	0.020	
-*Aralia elata*	20% H_2SO_4, 4 hrs.)		0.2073	0.061	
-*Aralia undulate*	LLE ($CHCl_3$, n=5)		0.0994	0.025	
-*Aralia cordata*	Rotary evaporation		0.6380	0.044	
	Dissolvation (MeOH)				
-*Crataegus pinnatifida*	Soxlet extraction (ether, to	Alltima C_{18} (250 mm x	0.532	1.4	[42]
	colorless)	4.6 mm, 5 μm)			
	Filtration	M.p. (MeOH : H_2O =			
	Rotary evaporation	87:13)			
	Dissolvation (MeOH)	R = 1 mL/min			
-*diospyros kaki*	Sonication (MeOH, 30	Shimadzu C_{18} column	0.17	2.2	[43]
	min. n=3)	(150 mm x 4.6 mm, 5	0.21		
	Filtration	μm)	0.22		
	Desiccation	M.p. (MeOH : 0.2%			
	Dissolvation (MeOH)	H_3PO_4 = 90:10)			
		R = 0.8 mL/min			
		λ = 220 nm			
-*Patrinia heterrphylla*	Optimization of extraction	Kromail ODS column			[44]
Xianxi Yulin	method (sonication, reflux	(150 mm x 4.6 mm, 0.5			
Root	and soxlet extraction)	μm)	0.54	0.07	
Stem	Acid hydrolysis	M.p. (MeOH : 0.05%	0.30	0.17	
Leaves	(sonication, 10% H_2SO_4, 4	CH_3COOH = 88:12)	0.15	0.13	
Whole plant	hrs., 50 min.)	1.0 mL/min	0.47	0.11	
Xianxi Taibai Province	LLE ($CHCl_3$)	λ = 225 nm			
Root	Rotary evaporation		0.56	0.09	
Stem	Dissolvation (MeOH)		0.31	0.10	
Leaves			0.16	0.19	
Whole plant			-	-	
Jinan					
Root			0.55	0.05	
Stem			-	-	
Leaves			-	-	
Whole plant			-	-	

Nanjing					
Root			0.32	0.03	
Stem			-	-	
Leaves			-	-	
Whole plant			-	-	
Henan Luoyang					
Root			0.30	0.07	
Stem			-	-	
Leaves			-	-	
Whole plant			0.20	1.00	
-*Fructus Mume*	Soxlet extraction (ether, 4 hrs.) Filtration Rotary evaporation Dissolvation (MeOH)	Shim-pack CLC-ODS column (150 mm x 6.0 mm) M.p. (MeOH : H_2O : HOAc : TEA = 85:15:0.04:0.02) R = 0.8 mL/min λ = 204 nm	0.5022 0.4965 1.0340 1.1327 0.6507 1.0092 0.3536 0.6195 0.5527 0.2363	1.01	[45]
-Leaves of *Photinia serrulata*	Optimization of extraction method (reflux (95% EtOH), soxlet extraction (95% EtOH), reflux (ether), soxlet extraction (ether)) Soxlet extraction (95% EtOH)	Zorbax ODS column (4.6 mm x 250 mm, 5 μm) M.p. (MeOH : 1% CH_3COOH = 88:12) λ = 215 nm R = 0.6 mL/min	0.6531	1.24	[46]
-*Swertia mileensis*	Reflux (MeOH, 60 min., n=3)	Phenomenex Luna C_{18}			[47]
Flowers		column (4.6 mm x 150	1.73	2.3	
Leaves		mm, 5 μm)	1.60		
Roots		M.p. (MeOH : H_2O =	0.35		
Stems		90:10)	0.67		
Whole plants		R = 1.0 mL/min	1.03		
		λ = 220 nm			
-*Swertia bifolia*	Sonication (MeOH, 30 min.)	Shim-Pack VP-ODS			[48]
Maerlian, Xichuan		(4.6 mm x 150 mm, 5	3.13	1.15	
S. bimaculata		μm)			

Jianshi, Hubei		M.p. (CH_3CN : H_2O =	3.27		
S. cincta		78:22)			
Luding, Xichuan		R = 1.0 mL/min	8.76		
S. davidii		λ = 215 nm			
Laifeng, Hubei			2.80		
S. diluta					
Jinchuan, Xichuan			5.52		
S. erythrosticta					
Maerlian, Xichuan			9.93		
Erlong, Xichuan			4.96		
S. kouitchensis					
Jianshi, Hubei			4.27		
Hefeng, Hubei			6.26		
S. macrosperma					
Luding, Xichuan			3.78		
Xuanxuan, Hubei			4.18		
S. nervosa					
Jianshi, Hubei			5.42		
Hefeng, Hubei			3.95		
S. przewalskii					
Baqiushan, Xichuan			4.39		
S. mussotii					
Erlong, Xichuan			12.2		
S. punicea					
Luding, Xichuan			6.32		
Jianshi, Hubei			7.74		
Halenia ellipica					
Kangding, Xichuan			3.15		
Lomatogoniopsis alpina					
Jinchuan, Xichuan			2.92		
Perilla frutescens (Leaves)	Cold extraction (EtOH, 40 mL, 24 hours) LLE (petroleum ether, $CHCl_3$ and n-BuOH) CC ($CHCl_3$ fraction, silica gel column 600 g, petroleum ether-acetone mixtures of increasing	Sperisob ODS (5 µm x 25 cm x 4.6 mm) M.p. (Acetonitrile : 1.25% H_3PO_4 = 86:14) R= 0.5 mL/min λ= 206 nm	0.1-0.2	0.9-11.8	[59]

	polarity)				
	NMR analysis				
-*Hedyotis diffusa*	Soxlet extraction ($CHCl_3$,	HiQ sil C_{18}V (4.6 mm x			[50]
Jiangxi	8 hrs.)	150 mm, 5 μm)	0.0650	1.3	
Liuzhou, Guangxi	Filtration	M.p. (MeOH : 20	0.0647		
Nanning, Guangxi	Rotary evaporation	mmol/L t-butyl NH_4Br :	0.0542		
Sanming, Fujian	Dissolvation (MeOH)	triethylamine =	0.0474		
Longyan, Fujian		90:10:0.02)	0.0585		
Jianzhou, Fujian		λ = 210 nm	0.0401		
Shaxian, Fujian		R = 0.3 mL/min	0.0284		
Hubei			0.0380		
-*Hedyotis diffusa*	Soxlet extraction (acetone,	Nucledure C_{18} column	1.06	4.28	[51]
	1 hr.)	(150 mm x 4.6 mm, 5			
	Filtration	μm)			
	Rotary evaporation	M.p. (20 mmol Na_3PO_4			
	Extract: NaOH: EtOH =	in H_2O : ACCN =			
	20:20:10.	55:45)			
	Centrifugation	λ = 220 nm			
	Collect Centrifuge	R = 1.0 mL/min			
	Neutralization (HCl, pH 5)				
	Centrifugation				
	Collect Centrifuge				
	Dissolvation (EtOH)				
-*Halenia elliptica*	Optimization for the	Phenomenex kromasic			[52]
Xiling, Qinghai	method of extraction	C_{18} column (4.6 mm x	0.32		
Datong, Qinghai	Optimization for the	250 mm, 5 μm)	0.24		
Huzhu, Qinghai	extraction solvent	M.p. (MeOH : H_2O :	0.45		
Huangzhong, Qinghai	Sonication (EtOH, 30 min.,	H_3PO_4 = 96:4:2)	0.29		
Langxuan, Xizang	n=3)	λ = 220 nm	0.29		
Bomi, Xizang		R = 1.0 mL/min	0.60		
Qushui, Xizang			0.69		
Bangda, Xizang			0.92		
Growth period					
Xiling					
offspring			0.34	0.02	
budding			-	-	
florescence			0.35	0.02	

fruit			0.32	0.04	
Huangzhong					
offspring			-	-	
budding			0.38	0.01	
florescence			0.45	0.04	
fruit			0.29	0.01	
-*Psidium Guava* Leaves	Optimization of extraction by fractional factorial design matrix (2^{5-1}) (temp., time, solvent vol., powder size, choice of solvent) Comparison of soxlet extraction with micro-wave assisted extraction Micro-wave assisted extraction (60-80 mesh powder, 10 mL EtOH, 80 ^{o}C, 10 min., n=3)	Lunar C_{18} column (250 mm x 4.6 mm, 5 µm) M.p. (0.05% H_3PO_4 : MeOH : ACCN = 11:56:33) λ = 210 nm R = 0.6 mL/min	0.92		[53]
-*Eriobotrya japonica*	Defatting with ether Reflux (EtOH, n=2) Decolorization (0.5% activated Carbon) Filtration Rotary evaporation C.C. (resin, H_2O, 90% EtOH) Collect EtOH fraction Rotary evaporation	Suntek C_{18} column (250 mm x 4.6 mm, 5 µm) M.p. (MeOH : H_2O = 94:6) λ = 220 nm R = 0.6 mL/min	0.96	3.46	[54]
-*Eriobotrya japonica*	Comparison of reflux and soxlet extraction. Comparison of ether and 95% EtOH. Soxlet extraction (95% EtOH, 6 hrs.) Filtration Rotary evaporation	Zorbax ODS column (4.6 mm x 250 mm, 5 µm) M.p. (MeOH : 1% H_2SO_4 = (88:12) R = 0.6 mL/min λ = 215 nm	0.394	1.34	[55]

	Dissolvation (MeOH)				
-*Diospyros kaki*	Sonication (MeOH, 30 min, n=2)	Shimadzu C_{18} column (4.6 mm x 150 mm, 5 μm) M.p. (MeOH : 0.2% H_3PO_4 = 90:10) R = 0.8 mL/min λ = 220 nm	0.17 0.21 0.22	2.20	[56]
-*Akebia trifoliate* Pingwu, Xichuan Bazhong, Xichuan Yaan, Xichuan Yibin, Xichuan Guangwyuan, Xichuan Zunyi, Guizhou Jingmen, Hubei	Sonication (50% EtOH, 1200 W, 30 min.) Filtration Sonication (10 min.) Filtration Rotary evaporation Acid hydrolysis (reflux, 2.5 mol/L H_2SO_4, 4 hrs., $CHCl_3$, 15 min.) LLE ($CHCl_3$) Rotary evaporation Dissolvation (MeOH)	Hypersil ODS column (4.6 mm x 250 mm, 5 μm) M.p. (In 0 min., H_2O: MeOH 25:75. In 10 min., 10:90. In 20 min., 5:95) λ = 215 nm R = 0.8 mL/min	0.413 0.307 0.596 0.543 0.436 0.302 0.500	1.02	[57]
Harvested at 2004-12 -*Eriobotrya japonica* Residue Harvested at 2005-02 -*Eriobotrya japonica* Residue	Sonication (95% EtOH, 1 hr.)	Waters Nova-Pak C_{18} column (3.9 mm x 300 mm) M.p. (MeOH : H_2O = 88:12) R = 0.8 mL/min λ = 210 nm	0.178 0.187 0.182 0.186	0.9 1.3 1.6 0.7	[58]
Grape skin Hetian Hetian White Munage (Kashgar) Red Munage (Kashgar)) Tulupan Tulupan Tulupan Tulupan Yili Yili	Cold extraction (MeOH, 30 min.) Sonication (15 min.) Dilution	Eclipes XDB C_{18} (4.6 mm x 150 mm, 5 μm) M.p. (MeOH : H_2O = 85:15) R = 1.0 mL/min λ = 220 nm	0.7613 0.9782 0.8370 1.0082 0.6560 0.9009 0.6569 0.9803 0.9860 0.6930	0.026 0.038 0.060 0.067 0.061 0.030 0.031 0.065 0.064 0.058	[59]

Anningqu			0.7042	0.043	
Shanshan			0.7145	0.048	
-Stem and leaf of *Lycopus lucidus*	Soxlet extraction (ether, to colorless) Suction filtration Rinse (petroleum ether, 6-8 ^{o}C, n=2) Rotary evaporation Dissolvation (MeOH)	C_{18} column (250 mm x 4.6 mm) M.p. (MeOH : H_2O : CH_3COOH = 89:11:0.2) λ = 205 nm R = 0.5 mL/min.	0.10035 0.12774 0.10834	0.23 0.97 0.50	[60]
-*Sambucus chinensis* Lindl January March May July September November	Acid hydrolysis (reflux, 60 mL 95% EtOH, 2 mL 20 % HCl, 2 hrs.) Filtration Neutralization (NaOH, pH 5) Standardization (EtOH) Dilution Centrifugation Dilution	Waters Symmetry C_{18} column (3.9 mm x 150 mm) M.p. (MeOH : H_2O = 88:12) R = 0.6 mL/min λ = 220 nm	0.029 0.034 0.041 0.038 0.026 0.031	1.32 0.92 2.30 1.76 2.13 1.42	[61]
-*Sambucus chinensis* Lindl Fengxin Yifeng Gaoan Wanzai Yuanzhou Province	Acid hydrolysis (reflux, 60 mL 95% EtOH, 2 mL 20 % HCl, 2 hrs.) Filtration Neutralization (NaOH, pH 5) Standardization (EtOH) Dilution Centrifugation Dilution	Waters Symmetry C_{18} (3.9 mm x 150 mm, 5 μm) M.p. (MeOH : H_2O = 88:12) λ = 220 nm R = 0.6 mL/min	0.041 0.035 0.038 0.029 0.034	2.30 1.13 1.42 1.15 1.26	[62]
-*Sambucus chinensis* Lindl. <u>Whole plant</u> Bozhou,Anhui Yongxing, Hunan Yichun, Jiangxi Xinjian, Jiangxi <u>Stem</u> Yongxing, Hunan	Soxlet extraction (95% EtOH, 8 hrs.) Rotary evaporation Dissolvation (MeOH) Dilution	Hypersil –ODS_2 column (4.6 mm x 250 mm, 5 μm) M.p. (MeOH : H_2O = 85:15) R = 0.8mL/min λ = 210 nm	0.03654 0.03672 0.06568 0.05688 0.03503	3.0	[63]

Leaf					
Yongxing, Hunan			0.06057		
Root					
Yongxing, Hunan			0.01670		
-Chinese Quince	Optimization for the extraction method (reflux, soxlet extraction, cold extraction, sonication) Sonication (95% EtOH, 4 hrs.) Filtration Rotary evaporation LLE ($CHCl_3$, H_2O, n=6) Rotary evaporation ($CHCl_3$) Dissolvation (MeOH)	Zorbax eliqse XPB C_{18} column (150 mm x 4.6 mm, 5 μm) M.p. (MeOH, H_2O = 80:20) R = 1 mL/min λ = 210 nm	0.2874	1.5	[64]
-*Fructus chaenamelis*	Reflux (MeOH, 1 hr.)	YMC C_{18} (4.6 mm x 250 mm, 5 μm) M.p. (ACCN : H_2O = 88:12) R = 0.8 mL/min ELSD			[65]
Zhangyang, Hubei			50.8	2.2	
XuanCheng, Anhui			75.1	2.3	
Beizhuan, Xichuan			82.5	2.8	
-*Canus officinalis*	Optimization of extraction method (soxlet extraction, reflux) Optimization of extraction solvent (90% EtOH, ether) Soxlet extraction (ether, 10 hrs.)	Phenomenex C_{18} column (250 mm x 4.6 mm, 5 μm) M.p. (MeOH : 0.5% CH_3COOH = 87:13) R = 0.8 mL/min ELSD			[66]
Xixia, Henan			0.0353	2.18	
Foping, Xiaxi			0.0188	1.36	
Taibai, Xiaxi			0.0275	1.92	
Xixia, Henan			0.0459	2.58	
Danfeng, Xiaxi			0.0487	2.76	
-*Hedyotis diffusa* Willd.	Soxlet extraction ($CHCl_3$, 8 hrs.) Filtration Rotary evaporation Dissolvation (MeOH)	Kromasi C_{18} 0DS (4.6 mm x 250 mm, 5 μm) M.p. (MeOH : H_2O = 82:18) λ = 210 nm R = 1.0 mL/min			[67]
Guangzhou, Guangdong			0.071	0.04	
Haikou, Hainan			0.103	0.27	
Gueilin, Guangxi			0.090	2.36	
-*Hedyotis corymbosa*					
Guangzhou, Guangdong			0.252	0.28	

Haikou, Hainan			0.373	1.33	
Gueilin, Guangxi			0.089	2.38	
-*Herba Hedyotis diffusae*	Reflux (EtOH, 90-95 ºC, 1 hr) Filtered Steam evaporation LLE ($CHCl_3$, NaOH, 10 min., n=3) Steam evaporation Dissolvation (m.p.)	Optimization of HPLC conditions Waters symmetry C_{18} (4.6 mm x 150 mm, 5 µm) M.p. (MeOH: ACCN: H_2O : EtCOOH = 72: 13: 15: 0.4) λ = 210 nm R = 1.0 mL/min Column temp. = 35 ºC	-	-	[68]
Weiyanting oral liquid	LLE (benzene, n=3) Steam evaporation Dissolvation (MeOH)	SB-C_{18} column (4.6 mm x 150 mm, 5 µm) M.p. (MeOH : H_2O = 85:15) R = 0.8 mL/min λ = 220 nm	0.46 0.44 0.48	3.90	[69]
Weiyanting capsules	Soxlet extraction (petroleum ether, to colorless) Reflux (benzene, 5 hrs.) Rotary evaporation Dissolvation (MeOH)		3.60 3.72 3.84	3.23	
Yishen Jiangu Tablets	Sonication (MeOH)	C_{18} column (4.6 mm x 150 mm, 5 µm) M.p. (ACCN : 0.1% H_3PO_4 = 67:33) R = 1.0 mL/min λ = 210 nm	11.56 12.31 12.86 7.74 10.23 10.38 10.09 10.62 10.26 9.54	0.27	[70]
Tiaozhibaogan granules	Sonication (MeOH, 20 min.)	Hypersil C_{18} column (4.6 mm x 150 mm, 5 µm) M.p. (MeOH : H_2O : acetic acid :	1.98 2.01 1.96	1.25 0.95 1.18	[71]

		triethylamine = 87:13:0.04:0.02) R = 1.0 mL/min λ = 210 nm			
Tianhusui Yugan tablets	Removal of capsule Reflux (EtOH, 1 hr.) Filtered Acid hydrolysis (reflux, 10 mL filtrate, 5 mL 20% HCl, 2.5 hrs.) Heating to evaporate EtOH (H_2O) LLE (CH_2Cl_2, n=3) Steam evaporation Dissolvation (MeOH)	C_{18} column (4.6 mm x 150 mm, 5 μm) M.p. (ACCN : 0.1% H_3PO_4 = 80:20) R = 1.0 mL/min λ = 210 nm	0.40 0.42 0.18 0.80 0.68 0.55 0.97 0.52 0.25 0.39	0.79	[72]
Sanliangban Yaojiu	Acid hydrolysis (reflux, petroleum ether, HCl, n=3) Steam evaporation Dissolvation (MeOH)	Hypersil ODS column (4.6 mm x 250 mm, 5 μm) M.p. (MeOH : H_2O = 90:10) R = 0.8 mL/min λ = 220 nm	7.23 ug/mL 9.76 ug/mL 9.84 ug/mL 1.30 ug/mL 1.50 ug/mL 2.91 ug/mL 2.64 ug/mL 6.85 ug/mL	2.2	[73]
Puguosuan capsules	Sonication (MeOH, 15 min.)	Eclipes XDB-C_{18} (4.6 mm x 150 mm, 5 μm) M.p. (MeOH: H_2O = 85:15) R = 1.0 mL/min λ = 220 nm	8.463 8.876 9.000	1.77 1.10 1.62	[74]
Chinese Flowering Quince Fruit Vinegar	-Flesh juice -Acid hydrolysate of flesh juice -EtOH extract of flesh juice -Iso-propanol extract of flesh juice -Iso-propanol extract of dried fruit	Nucleodur100-5, C_{18} EC column (4.6 mm x 250 mm, 5 μm) M.p. (MeOH: H_2O = 95:5) R = 1.0 mL/min λ = 210 nm	0 0 1 0.995 1.145	-	[75]

	-EtOH extract of dried fruit		1.250		
	-50% EtOH extract of powdered dried fruit		0		
	-50% EtOH extract of residue fruit		0		
	-concentrate extract of 50% EtOH extract of fruit		0		
	-acid hydrolysis of concentrate extract of 50% EtOH extract of fruit		0		
	-filtrate of fermentation of fruit		0		
	-fruit vinegar (fermentation)		0.260		
	-fermentation extract of fruit vinegar (fermentation)		0.482		
	-filtrate of fermentation extract of fruit vinegar (fermentation)		0.448		
	-Hong Qu vinegar (fermentation)		0.402		
	-Vinegar		0		
	-Alcoholic fermentation of flesh juice		0		
	-Fermentation of flesh juice + sugar		0		
	-Fermentation of flesh juice + pineapple		0		
	-Filtrate of fermentation of flesh juice + pineapple		0		
	-Fermentation of flesh juice + apple		0.02		
	-Fermentation of apple vinegar		0		
Sterilized Powder of Oleanolic acid Nanoparticles (OLA-NP) for Injection.	Dissolvation (MeOH)	Agilent C_{18} (4.6 mm x 250 mm, 5 µm) M.p. (MeOH : H_2O :: EtCOOH : $(C_2H_5)_3N$ =	95.42 97.18 95.57 96.70	1.016	[76]

		900: 100: 0.2: 0.3) R = 1.0 mL/min λ = 210 nm	95.02 95.45 99.49 97.10 91.40 95.21		
Compound Liuyuexue Granules	Reflux (EtOH, 90-95°C, 1 hr.) Filtered Steam evaporation LLE ($CHCl_3$, H_2O, NaOH 10 min., n=2) Steam evaporation ($CHCl_3$ layers) Dissolvation (m.p.)	Waters symmetry C_{18} (4.6 mm x 150 mm, 5 µm) M.p. (MeOH : ACCN : H_2O :: EtCOOH = 72:13:15:0.4) R = 1.0 mL/min λ = 210 nm	0.01321 0.01462 0.01458 0.01503 0.01392	3.30	[77]
Tongmai Huoluokang Granules	Reflux (EtOH, 30 min., n=3) Filtered Steam evaporation Dissolvation (MeOH)	Kromasil-C_{18} (4.6 mm x 150 mm, 5 µm) M.p. (MeOH : H_2O = 90:10) R = 1.0 mL/min λ = 220 nm	0.261 0.253 0.272	1.10	[78]
Mitracarpus scaber	Cold extraction (EtOH, 72 hours) Filtered Rotary evaporation CC (silicagel 0.062-0.2 mm, 2.2 cm x 20 cm) MPLC (silica gel, Lichroprep Si 60 Merck, omnifit glass column OM 6427 15 mm x 750 mm, with Gilson SC-Type Pump, toluene,, touen-E.A. (90:10), toluene-E.A.-MeOH (80:18:2). Rotary evaporation	SPHERISORBC_{18} ODC column (5µm x 4.6 mm x 25 cm) M.p. (ACCN:H_2O = 85:15) R=0.6 mL/min λ= 215 nm	0.0043	2.13-3.72	[79]
-*Swertia mussotii*	Extraction (100% EtOH)	Phenomenex kromasil			[80]

Xining		C_{18} column (5 μm x 250	0.309	0.08	
Nanshan		mm x 4.60 mm)	0.253	0.02	
Datong			0.249	0.12	
Huangzhong			0.307	0.10	
Jiangda			0.503	0.05	
Yushu			0.194	0.34	
Chenduo			0.256	0.27	
Nangqian			0.288	0.10	
Rosette stage			0.249	0.34	
Bud stage			0.397	0.63	
Flowering stage			0.260	0.35	
Fruiting stage			0.253	0.02	
Sanduoxiao Tea Bag	Soxlet extraction (MeOH,	Kromasil-C_{18} (5.0 nm x	0.117	1.59	[81]
	6 hrs.)	200 μm, 5 μm)	0.106	2.01	
		M.p. (MeOH : 0.5%	0.125	1.78	
		EtCOOH = 84: 16)			
		R = 1.0 mL/min			
		λ = 210 nm			
-*Perilla Frutescens*	Reflux (MeOH, 80 ºC ,	Spherisob ODS, 5 μm x	-	-	[82]
	n=2)	25 cm x 4.6)			
	Filtered	M.p. (ACCN: 1.25%			
	Rotary evaporation	H_3PO_4 = 86:14)			
	Dissolvation (MeOH)	R= 0.5 mL/min			
		λ= 206 nm			
-*Eriobotrya japonica*	Sonication (EtOH, 2 hours,	ODS C_{18} column (250 x			[83]
Flowers	30 min., n=2)	4.6 mm, 5 μm)	0.045	8.89	
Petal	Rotary evaporation	M.p. (MeOH : 0.03	0.012	8.33	
Sepal	Dissolvation (MeOH)	mol/L phosphate buffer	0.68	7.35	
Pistill + stamen		(pH 2.8) = 88:12)	0.24	8.33	
Peduncle		R= 1.0 mL/min.	0.57	7.02	
		λ = 210 nm			

Results and Discussion

A method for determination of OA from RAB was developed, optimized and validated. In addition, a method for determination of OA from natural products was developed by generalization of method of determination of OA from RAB and the results were summarized in Figure 43. Different natural products have different OA content. OA content determined by other methods was summarized and tabulated (Table 17). However, their OA contents were analyzed by different extraction and HPLC procedures. In

addition, some of them were not “really” optimized. Moreover, some optimized extractions were developed for the purpose of extracting maximum content of OA [8,9] but not for purpose of determination. For standardization, using one experimental procedure to determine OA contents for all natural products is required for fair comparison.

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