Heliyon 9 (2023) e18509

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

A multi-analyte screening method for the rapid detection of illicit adulterants in dietary supplements using a portable SERS analyzer

Serena Rizzo [a,b,c], Yannick Weesepoel [a], Sara Erasmus [d], Joost Sinkeldam [a], Anna Lisa Piccinelli [b], Saskia van Ruth [a,d,*]

[a] Wageningen Food Safety Research, Wageningen University and Research, P.O. Box 230, 6700 AE, Wageningen, the Netherlands
[b] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Salerno, Italy
[c] PhD Program in Drug Discovery and Development, University of Salerno, Via Giovanni Paolo II 132, Fisciano, SA, 84084, Italy
[d] Food Quality and Design Group, Wageningen University and Research, P.O. Box 17, 6700 AA, Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Adulteration
Food fraud
Food safety
Health supplements
Portable device
Surface-enhanced Raman spectroscopy

ABSTRACT

The popularity and number of dietary supplements on the health market have experienced an unprecedented boost in recent years. Simultaneously, their increased use has been accompanied by an increase in acute intoxication cases linked to the adulteration of these products with illicit and undeclared substances. In this study, a SERS-based screening methodology was developed to rapidly detect illegally added pharmaceutically active substances to dietary supplements. A portable analyzer and silver printed-SERS substrates were used to enhance the signal, requiring less than 20 min of sample preparation prior to the analysis. The method was successful in the qualitative identification of eleven out of twenty-three illicit adulterants in the dietary supplements; it could detect the target compounds at realistic adulteration levels (0.1–5.0% w/w), demonstrating the potential of SERS-based methodologies for forensic rapid screening applications. The developed method is quick, easy to use, requires no skilled technicians and little sample preparation, and allows in-situ analyses. For these reasons, it is suitable for quick screening to be performed by inspectors at customs. Moreover, the low specificity of spectroscopic methods, to which SERS belongs, would benefit the detection of newly synthesized analogues of the target adulterants, which would otherwise be more difficult using common mass spectrometry methods in absence of reference standards.

1. Introduction

The consumption of dietary supplements (DS) has dramatically increased all over the world in the last twenty years [1]. The reason is that the formulation of these products often includes plant extracts advertised as “natural” ingredients so many consumers perceive them as healthier and safer than conventional pharmaceutical products [2]. Moreover, dietary supplements are readily available in retail and virtual outlets as they are legally considered foods, both in the European Union (EU) and United States (US) under the Directive 2002/46/EC [3] and the Dietary Supplement Health and Education Act (DSHEA) of 1994 [4], respectively. Hence, they are less controlled in the retail market than pharmaceutical products, which offers opportunities to motivated offenders to illegally inflate

* Corresponding author. Wageningen Food Safety Research, Wageningen University and Research, P.O. Box 230, 6700 AE, Wageningen, the Netherlands
E-mail address: saskia.vanruth@wur.nl (S. van Ruth).

https://doi.org/10.1016/j.heliyon.2023.e18509
Received 17 April 2023; Received in revised form 18 July 2023; Accepted 19 July 2023
Available online 20 July 2023

their effects. In fact, the increasing availability and use of dietary supplements have been accompanied by an increasing frequency of adulteration of these products with different illicit and undeclared substances, such as pharmaceutical drugs or analogues and botanical markers of unauthorized plants, added to provide rapid effects and increase sales [5,6]. Pharmaceutical drugs include appetite suppressors, diuretics, and laxatives in weight-loss supplements, phosphodiesterase type-5 inhibitors (PDE5I) in sexual performance enhancement supplements, and anabolic steroids in muscle building supplements [7,8]. An additional challenge concerns the use of structural analogues of those substances, for which no pharmacological studies are available. Newly synthetized analogues of pharmaceutical adulterants could be even more threatening to human health as no knowledge on their toxicity and side effects is available [9–13]. Unauthorized plants can also be used, which includes vegetal matrices with well-known adverse effects [14]. The presence of such adulterated products in the marketplace is a worldwide problem and their consumption poses major health risks to consumers [15,16]. To enable the health authorities of the EU member states to take immediate action, warn consumers, and withdraw certain products from the market, the Council of the member states founded the Rapid Alert System for Food and Feed (RASFF) in 2002 [17]. The RASFF is a network that provides the supervisory authorities with a rapid and efficient mechanism for exchanging knowledge on the notifications issued by the different EU member states every time a food presents a serious risk to public health due to contamination, adulteration, or lack of framing in laws. Since 2002, the number of RASFF notifications in the product category "dietetic products, food supplements and fortified foods" has steadily increased (Fig. 1), positioning as second most notified category of the year 2020 and most notified category of both the years 2019 and 2018 [18–36]. Likewise, the US Food and Drug Administration (FDA) is responsible for managing these products, their release on the market as well as recalls and withdrawals from it. Despite the potentially serious health risks, the size and spread of the problem is not well documented.

Traditional analytical methods for the determination of adulterants in dietary supplements are based on liquid chromatography combined with mass spectrometry (LC-MS); these methods are time-consuming, costly and depend on laboratory-based analyses [37, 38]. Hence, for a better overview of the food fraud landscape on dietary supplements, a rapid screening methodology that could be applied on site would be very beneficial. Surface-enhanced Raman spectroscopy (SERS) has recently attracted a lot of attention for the detection of food adulterations as it meets these requirements [39]. It is based on the reciprocal interaction between an analyte and a metallic substrate, which consists in the transfer of electrons in the ground and excited states of the molecule–substrate system. The transfer occurs in the process of metal–molecule bond formation and is crucial to detect the intrinsic fingerprint of the analyte and amplify the Raman signal, making this technique sufficiently sensitive to detect single molecules on a surface [40]. The advantages of this methodology are its simplicity, high sensitivity, rapidity, low cost, and in-situ sampling and monitoring [41]. The technique is also relatively insensitive towards the pharmaceutical excipients used during the manufacturing of DS, which is a great advantage for the detection of single, specific adulterants [42]. Limitations of SERS include the fact that the adsorption of the analytes onto the SERS substrate is necessary, the selectivity of the substrates differs depending on the given analyte, and the substrates cannot be reused. Despite its limitations, the enhanced sensitivity of SERS compared to Raman spectroscopy has made it an interesting technique to detect a wide range of analytes, including illicit adulterants [43]. Moreover, the use of nanoparticles deposited on a surface (or printed-SERS substrates), instead of the most commonly used nanoparticles in colloidal solutions, provides reasonable enhancements as the particles aggregate during the deposition process, improving SERS signals. On the other hand, colloidal solutions suffer from instability over time and demanding storage conditions [44].

The aim of the present study was to explore the use of SERS for the qualitative screening and detection of illicit adulterants in DS using portable devices, with the goal of developing a rapid screening method suitable for this purpose, in collaboration with the Dutch Food and Consumer Product Safety Authority. This includes an appropriate evaluation of any interferences due to the presence of plant-based extracts and other ingredients present in the formulation of DS as well as the setting of detection ranges for the studied adulterants. For this purpose, a portable SERS analyzer and printed-SERS substrates were used. To the best of the authors' knowledge, this is the first study that attempts to develop a multi-analyte screening method for the rapid detection of illicit adulterants in dietary supplements using a portable SERS analyzer. The authors could only find studies reporting on the development of analytical methods using LC-MS systems [8], coupling Raman with chromatographic techniques which are not suitable for in situ analyses [45,46], or detecting specific classes of adulterants only [47], but not rapid screening methods which can detect different classes of adulterants at the same time using portable SERS devices.

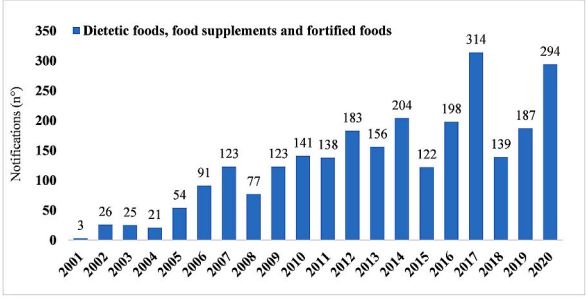


Fig. 1. Total number of Rapid Alert System for Food and Feed (RASFF) notifications in the product category "Dietetic foods, food supplements and fortified foods" from 2001 to 2020.

2. Materials and methods

2.1. Experimental approach

Twenty-three pharmaceutically active adulterants were used in the study, and their Raman activity examined using a benchtop FT-Raman spectrometer and a portable SERS analyzer. Those that were SERS active were used for the subsequent steps of method development. Then, a spectral library was created using the software associated to the portable instrument to verify the existence of any similarities between the SERS spectra of the target adulterants by applying basic statistical matching. Afterwards, a simple dilution and pre-concentration step of the analytes was developed and optimized before the instrumental analysis. The developed procedure was applied to the SERS analysis of different concentrations of each adulterant mixed with a representative mixture of excipients (range 0.1–50.0% w/w) in order to estimate the detectability range of each adulterant; the same was done by testing low concentrations of the adulterants mixed with eighteen commercially sourced DS with claims to reduce weight, enhance sexual performances, and build muscles (range 0.1–5.0% w/w) to test the applicability of the developed procedure in real conditions of adulteration and set the limit of identification (LOI) of each adulterant.

2.2. Chemicals and standards

Analytical grade ethanol (EtOH), acetone (Me_2CO), and acetonitrile (MeCN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (H_2O, 18 MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, USA).

The following reference standards of the twenty-three target adulterants were purchased from Biosynth International (Naperville, IL): acetildenafil (ACD), amino tadalafil (ATAD), homo sildenafil (HSIL), DL-5-hydroxytryptophan (HTP), piperin (PIP), tadalafil (TAD), thiosildenafil (TSIL), vardenafil hydrochloride (VAR), xanthoanthrafil (XAN), and Sigma-Aldrich (St. Louis, MO, USA): 4-androstene-3,17-dione (AND), 1,3-dimethylamylamine (DMAA), 2-dimethylaminoethanol (DMAE, ≥99.5%), 1,3-dimethylbutylamine (DMBA, 98%), 2,4-dinitrophenol (DNP), fluoxetine hydrochloride (FLU), melatonin (MEL, ≥98%), phenethylamine (PEA, 99%), phenolphthalein (PNP), sibutramine (SIB), sildenafil citrate (SIL), synephrine (SYN), vinpocetine (VIN), yohimbine hydrochloride (YOH, ≥98%). They were selected based on alerts from the RASFF portal.

The following excipients were provided by Sigma-Aldrich (St. Louis, MO, USA): arabic gum from the acacia tree, calcium hydrogen phosphate, carboxymethylcellulose sodium salt, cellulose microcrystalline, hydroxypropylmethylcellulose, lactose, magnesium stearate, methyl cellulose, polyvinylpyrrolidone, silicon dioxide, starch, stearic acid, and titanium oxide.

2.3. Commercial samples

Eighteen commercial DS samples were sourced and included nine weight-loss and energy boosting supplements (W1-9) and nine sexual and sport performance enhancement supplements (S1-9). The samples were purchased through different online e-commerce. One of the weight-loss and energy boosting supplements was formulated as powder, four as capsules, and four as tablets. Three of the sexual and sport performance enhancement supplements were formulated as tablets and six as capsules. All the samples were manufactured in different countries of the European Union and the United Kingdom. The excipients listed on the label of the samples were mainly microcrystalline cellulose, magnesium stearate, hydroxypropyl methylcellulose, and silicon dioxide for both capsules and tablets. Among the weight-loss and the energy boosting supplements, the principal plant-based ingredients claimed on the label were Garcinia cambogia fruit extract (*Garcinia gummi-gutta*), green tea (*Camellia sinensis*) leaf extract, and green coffee (*Coffea robusta*) dried extract. Among the sexual and sport performance enhancement supplements, the principal plant-based ingredients claimed on the label were maca root (*Lepidium meyenii*), gokshur (*Tribulus terrestris*), ginkgo (*Ginkgo biloba)*, ashwagandha (*Withania somnifera*), rhodiola (*Rhodiola rosea*), stone pine (*Pinus pinea*), fennel seed (*Foeniculum vulgare*), fenugreek (*Trigonella foenum-gra cum*), psyllium husk powder (*Plantago ovata*), aloe leaf extract (*Aloe vera*), black chia seed powder (*Salvia hispanica*), and flaxseed powder (*Linum usitatissimum*), broccoli dried extract (*Brassica oleracea*), ginseng dried extract (*Panax ginseng*). Ten of the supplements also included different vitamins (B5, B6, B9, B12, C, D3, E, K) and minerals (calcium, chromium, magnesium, selenium, zinc) in their formulation. The average weight of the supplements was 1000 mg with supplements ranging from a minimum weight of 200 mg up to a maximum of 1800 mg. The recommended dosage for all the supplements ranged from 2 to 4 tablets/capsules per day.

2.4. Sample preparation

2.4.1. Artificially adulterated excipients mixture

The detectability range of each SERS active adulterant was assessed by measuring eight levels of adulteration, in the range from 0.1% to 50.0% w/w, within a basic formulation of DS (excipients only; no other additional ingredients were added). In detail, a mixture of excipients was prepared to be used as filling material and it was prepared as follows: microcrystalline cellulose (95.0% w/w), magnesium stearate (3.0% w/w), silicon dioxide (1.0% w/w), hydroxypropyl methylcellulose (0.5% w/w), and titanium oxide (0.5% w/w). The excipients were selected to reflect a composition of excipients as representative as possible of the real formulations by observing the composition of the excipients on the labels of various DS present on the market. The final percentage of each component of the mixture was chosen based on the experience of the researchers who have worked on the project. No plant-based extracts were used during this step since it was not possible to choose a representative set of plant-based ingredients due to the enormous variability in their composition within the formulation of DS.

2.4.2. Artificially adulterated DS samples

Eighteen commercial DS were used as filling materials for the preparation of five low levels of adulteration, in the range from 0.1% to 5.0% w/w, to test the applicability of the SERS method to the routine screening of suspected fraudulent DS and estimate the limit of identification (LOI) of the target adulterants, which is the lowest concentration for which the identification criteria are met. To do so, the target adulterants were divided in two groups based on the frequency of their detection as adulterants in the considered categories of DS; thus, FLU, MEL, PEA, SYN, and VIN were used to adulterate the nine weight-loss and energy boosting supplements (W1-9) while ACD, HSIL, PIP, SIL, TSIL, and VAR were used to adulterate the nine sexual and sport performance enhancement supplements (S1-9).

The mixing of all the excipients (necessary for the preparation of the mixture) as well as the preparation of the adulteration levels of all the target analytes were carried out using the geometric dilution method, commonly used in the pharmaceutical industry to produce formulations with a low content of active ingredients [48]. It consists of the gradual addition of equal portions of an excipient to the pharmacologically active ingredient, taking care to double the present quantity each time, to achieve an equitable distribution of the active ingredient particles within the mixture. During the analysis of each batch, both 100% w/w (pure standard) and 0% w/w (mixture of excipients, blank sample) were also tested.

2.5. Raman and SERS spectroscopy

2.5.1. Benchtop FT-Raman spectrometer

The Raman activity of the pure target adulterants was tested on the solid or liquid materials using a benchtop RAM II FT-IR Raman module (Bruker Nederland B·V., Leiderdorp, The Netherlands) coupled to a FT-IR VERTEX 70 spectrometer. The spectrometer was equipped with a 1064 nm laser and the spectra were recorded with a resolution of 4 cm^{-1} in the range of 50–3600 cm^{-1}, resulting in an average of 32 consecutive scans. The laser output power was set at 300 mW. Three spectra were recorded for each sample and averaged in the following step of data analysis. Spectral data were acquired using OPUS software (version 7.2, Bruker).

2.5.2. Portable SERS analyzer

The SERS analyses were performed using a Metrohm Instant SERS analyzer (MISA) (Metrohm, Laramie, WY, USA) equipped with a 785 nm $\pm$ 0.5 nm laser and an Orbital Raster Scan (ORS™) technology where a focused laser spot (30 μm in diameter) is continuously rasterized over a region of ≈2 mm in diameter on the substrate, resulting in a higher sample coverage and better averaging. Spectra were recorded with a resolution of 8 cm^{-1} (FWHM) in the range of 400–2300 cm^{-1} obtaining an average of three successive scans. The laser output power was 100 mW. Three spectra were recorded for each sample and averaged in the following step of data analysis. Silver printed-SERS (*p*-SERS) substrates and a specific *p*-SERS Attachment (Metrohm, Laramie, WY, USA) were used to carry out the SERS measurements. Gold *p*-SERS substrates were also tested for adulterants which were silver *p*-SERS inactive. Daily calibrations of the instrument were performed using the appropriate calibration standard (ASTM 1840 reference sample). Spectral data were acquired using the instrument-associated MISA Cal software (Metrohm) with the following standard operating procedure: Smart Acquire mode on, laser power level set to 5, auto integration on, averages of three successive scans, and raster on.

2.6. SERS procedure

Each artificially adulterated DS sample was processed as follows: 10 mg of each sample were first weighed into an Eppendorf tube and then diluted with 100 μL of EtOH to prepare a super-concentrated solution of the analyte. EtOH was used as dilution solvent for all the studied adulterants. Each sample was then vortexed for 20 s and centrifugated at 806,400 RCF for 2 min. Afterwards, 10 μL of the supernatant were pipetted onto the silver *p*-SERS strip and left to dry for 15 min after which the instrumental analysis commenced. Measurements were conducted in triplicates.

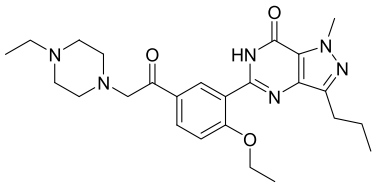
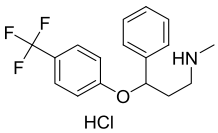
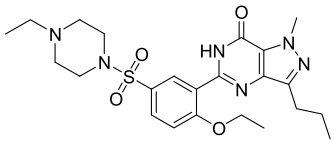
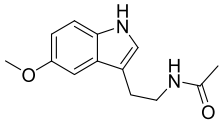
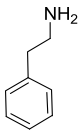
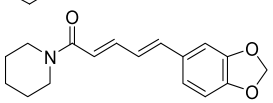
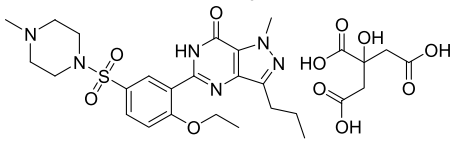
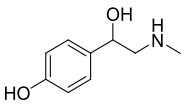
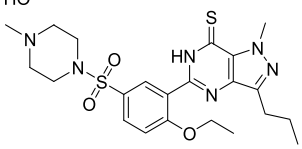
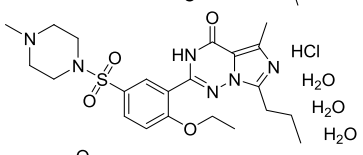
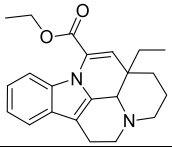
2.7. Spectral library creation and identification strategy

The MISA identification of unknowns is carried out by correlating a sample spectrum with the spectra of the library. Therefore, it was first necessary to build a spectral library of adulterants through the libraries section of the MISA Cal software. The library was then used for the screening of artificially adulterated samples of DS. All the spectra of the library were collected by diluting the pure adulterants in EtOH, at a concentration of 1 mg mL^{-1}. The software is equipped with a correlation algorithm for spectral comparison to identify an unknown spectrum against a spectral library. The library is selected for matching when the operating procedure is created. The software searches the library and returns a Hit Quality Index (HQI) indicating the level of correlation within a user-defined threshold. When analyzing a sample, the spectrum of the sample will then be compared with all the spectra of the selected library. The measured sample will be identified as one of the library samples and displayed as the 'Identification Result'. If the sample does not match a library, 'No Results' will be displayed. Moreover, the Confusion Matrix tab of the libraries section allows for evaluation of the accuracy of the classification. The software warns the user about the presence of any matches between the spectra of the library through three Warning categories, highlighted by the three colours: red (match score >0.95), orange (match score >0.85), and yellow (match score >0.75). Similarity scores below 0.75 are not highlighted as they are not considered warning scores.

2.8. Data analysis

Data analysis was performed using the instrument-associated MISA Cal software. The HQI matching was used to identify an un-

Table 1
Chemical structures, therapeutic daily doses, and European Commission (EC) status regarding the use of the Surface-enhanced Raman spectroscopy (SERS) active adulterants in dietary supplement formulations.

Adulterant	EC status	Chemical structure	Therapeutic daily doses (mg)
Acetildenafil	Forbidden		–
Fluoxetine hydrochloride	Forbidden		10–60
Homo sildenafil	Forbidden		–
Melatonin	Allowed		0.5–5
Phenethylamine	Forbidden		100–500
Piperin	Allowed		5–30
Sildenafil citrate	Forbidden		25–100
Synephrine	Allowed		30
Thiosildenafil	Forbidden		–
Vardenafil hydrochloride	Forbidden		5–20
Vinpocetine	Allowed		5–40

known spectrum. The correlation coefficient HQI for the unknown scan compared to the library spectrum is calculated using the least square dot product of the mean centred unknown spectrum and the library spectrum, represented by **Equation (1)**:

$$\mathrm{HQI} = \frac{(\mathrm{Library} \times \mathrm{Unknown})^2}{(\mathrm{Library} \times \mathrm{Library})(\mathrm{Unknown} \times \mathrm{Unknown})} \quad (1)$$

The HQI ranges from 0 to 1, with 0 representing no match and 1 representing a perfect match.

A second check of the data analysis was also carried out using an in-house script developed using R software version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) and RStudio version 5501.9.1.0. The R packages prospectr_0.2.0, signal_0.7–6, Rtools_4.0.3.0, and pracma were used. The statistical correspondence scores between Raman spectra of the eleven target adulterants and the artificially adulterated DS samples at different concentration levels were calculated as Pearson's correlation between the maximum/minimum intensity value of the reference peak (first derivative) and the values at the corresponding wavelength points of each unknown spectrum, using a second order filter. Subsequently, for each reference spectrum of the first derivative, the spectral features were identified using a generic peak-finder algorithm (pracma). Match scores range from 0 (no match) to 1 (perfect match). However, score values below 0 were also observed and interpreted as "absence of any similarity".

3. Results and discussion

3.1. Evaluation of the Raman activity of the target adulterants

3.1.1. Raman spectroscopy

The Raman activity of pure powdered (n = 19) and liquid (n = 4) adulterants (Table S1) was first tested using a benchtop FT-Raman instrument; the aim was to establish if the selected adulterants were Raman active before proceeding to study the improvement of the signal induced by their interaction with the *p*-SERS substrate. Aside from AND, DMAA, DMAE, DMBA, TSIL, VAR, and XAN, all the target analytes exhibited a characteristic Raman shift when tested with the benchtop FT-Raman instrument, albeit at very low intensities.

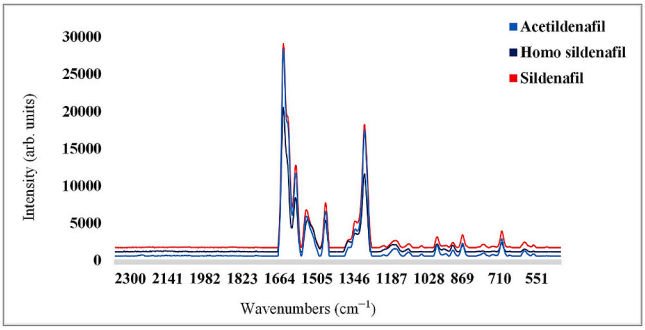
3.1.2. Surface-enhanced Raman spectroscopy (SERS)

As the aim of the study was to develop a rapid screening method using a portable analyzer, the SERS activity of the target adulterants was further investigated using the MISA device. The use of silver *p*-SERS substrates provided enhancement factors ranging from 10^3 to 10^4 only for eleven target adulterants. Table 1 shows the chemical structures, therapeutic daily doses, and European Commission (EC) status of the eleven SERS active adulterants. The enhancement of the Raman scattering for the molecules adsorbed onto the metal surface is due to the optical electric field enhancement produced by the interaction of the molecules with the silver particles [44]. Regarding the remaining SERS inactive adulterants, the SERS inactivity observed on the benchtop instrument was confirmed for AND, DMAA, DMAE, DMBA, and XAN while for ATAD, DNP, PNP, SIB and TAD the SERS inactivity is probably due to a lack of interaction of the analytes with the silver *p*-SERS substrate. HTP and YOH resulted SERS active albeit with a low signal intensity; solubility tests demonstrated that their low SERS activity was due to their poor solubility in the selected dilution solvent (EtOH) (see section 3.5). For this reason, HTP and YOH were excluded from the method.

Additional tests were also carried out on the latter five adulterants using gold *p*-SERS substrates; these tests further confirmed the inactivity of the tested analytes. Table S1 shows the comparison between FT-Raman and SERS activity of the twenty-three selected adulterants.

3.2. Evaluation of the Raman activity of the excipients

The Raman activity of the excipients was tested to determine the existence of any interferences between them and the target adulterants. This evaluation was necessary since the excipients are the basic ingredients of the main pharmaceutical forms used to



Library Sample	Match	Match Score
Acetildenafil	Sildenafil	0.99
Acetildenafil	Homo sildenafil	0.98
Homo sildenafil	Sildenafil	0.97

Fig. 2. Surface-enhanced Raman spectroscopy (SERS) spectra of acetildenafil, homo sildenafil, sildenafil, and library match scores among the three adulterants obtained by the MISA Cal software.

formulate dietary supplements (capsules and tablets). None of the tested excipients exhibited any pronounced Raman activity, meaning that the basic ingredients of all DS formulations did not interfere with the detection of the target adulterants. Fig. S1 shows the Raman spectra of all the tested excipients; SERS spectra were not included since none of the excipients was dissolved by the dilution solvent (EtOH), which is why SERS spectra of the excipients correspond to the spectrum of EtOH.

3.3. Spectral library and identification results

A SERS spectral library of the eleven selected adulterants was first built and then used to screen all the artificially adulterated DS samples; before doing so, it was necessary to establish the existence of any similarities between the spectra of the target adulterants to prevent the software from providing false assignments when screening the samples. The software showed three red match scores between the target adulterants due to match scores of 0.99 between ACD and SIL, 0.98 between ACD and HSIL, and 0.97 between HSIL and SIL. This led to the conclusion that the spectra of these three adulterants can be considered indistinguishable. Fig. 2 shows SERS spectra of ACD, HSIL, and SIL as confusion matrices of the library. Although the unfeasibility of distinguishing these three adulterants might seem like a drawback of the procedure, it is not since they belong to the same chemical-pharmacological class which is used for the same fraudulent purpose in sexual performance enhancement DS. Therefore, it is not a huge issue that the algorithm is not able to discriminate them. Rather, this could benefit the procedure since there is a chance that these adulterants could be added together within the same formulation. This would then lead to an increase in the intensity of the signal by superimposing the intensities of the single analytes. No orange and yellow matches were present among the spectra of the other adulterants of the library, indicating the absence of any other warning similarities and therefore, the risk of false positives.

3.4. Optimization of the SERS procedure

Before proceeding to the screening of the artificially adulterated DS samples, the SERS procedure was carefully optimized to select the optimum solvent to dilute the adulterants of interest and its appropriate amount. Solubility tests were carried out to select the best dilution agent for the screening of the selected adulterants; five different solvents: H_2O, EtOH, Me_2CO, MeCN, and the mixture EtOH:H_2O 50:50 v/v were tested. Among the tested solvents, EtOH was established as the most suitable solvent to dilute all the adulterants except for HTP and YOH, which showed good signals only if diluted with H_2O. However, H_2O was discarded because unsuitable in real scenarios of DS adulterations; in fact, additional tests showed that when used to dilute real formulations of herbal DS, H_2O brings into solution substances that interferes with the analytes detection. The mixture EtOH:H_2O 50:50 v/v was discarded because the results obtained from EtOH (individually used) showed to be better, in terms of signal enhancement, than those obtained from the mixture. Moreover, all the selected excipients were SERS inactive when diluted with EtOH. For these reasons, EtOH was selected as dilution solvent of the procedure. Subsequent tests were carried out to determine the amount of solvent to be used when performing the screening of artificially adulterated DS. To do so, three different amounts of EtOH, which are 0.1, 0.5 and 1 mL, were selected. It is important to mention that for this step of choosing the correct amount of solvent, the solubility limits of the selected compounds were not considered as the purpose of the present study was not to develop a quantitative screening method, which would have required not exceeding the limits of solubility of the selected compounds. The aim was instead to develop a rapid screening method providing a rapid pass/fail answer regarding the presence of the selected adulterants in DS using a portable device. Any positive sample must be subjected to further investigations to confirm the presence of the suspected adulterants and establish its amount; LC-MS methods are currently used for this purpose.

3.5. Screening of artificially adulterated DS samples

3.5.1. Analysis of artificially adulterated excipients mixture

To estimate a detectability range, eight levels of adulteration in the range of 0.1–50.0% w/w were prepared for each analyte using

Table 2
Detected ranges of artificially adulterated and comparison of Person's correlation scores between MISA Cal and R software.

Adulterant	MISA Cal software		R software	
	Detected range (% w/w)	Library match score	Detected range (% w/w)	Library match score
Acetildenafil[a]	0.1–50.0	0.99–0.99	0.1–50.0	1.00–1.00
Fluoxetine hydrochloride	0.5–50.0	0.76–0.96	0.5–50.0	0.91–0.99
Homo sildenafil[a]	0.1–50.0	0.99–0.99	0.1–50.0	1.00–1.00
Melatonin	0.5–50.0	0.79–0.98	0.1–50.0	0.75–0.99
Phenethylamine	0.5–50.0	0.78–0.99	0.5–50.0	0.95–1.00
Piperin	2.5–50.0	0.89–0.98	0.5–50.0	0.81–0.95
Sildenafil citrate[a]	0.1–50.0	0.99–0.99	0.1–50.0	1.00–1.00
Synephrine	2.5–50.0	0.73–0.99	1.0–50.0	0.72–1.00
Thiosildenafil	0.1–50.0	0.98–0.99	0.1–50.0	1.00–1.00
Vardenafil hydrochloride	0.1–50.0	0.93–0.99	0.1–50.0	0.98–1.00
Vinpocetine	0.1–50.0	0.95–0.99	0.1–50.0	0.92–0.99

[a] Surface-enhanced Raman spectroscopy (SERS) spectra of acetildenafil, homo sildenafil, and sildenafil were indistinguishable.

the mixture of excipients as filling material. The concentration range was chosen to cover the therapeutic range of the adulterants of interest. In detail, a calculation of the hypothetical concentration levels of adulteration was made, according to the active therapeutic doses of the target adulterants when added to formulations ranging from 100 to 2000 mg per unit to select the concentration range to test (Table S2). Then, the entire concentration range of all the target adulterants was analyzed using the portable SERS analyzer solo and it showed to be able to detect the target adulterants covering the entire concentration range (0.1–50.0% w/w) with correlation scores ranging from 0.93 to 0.99 for ACD, HSIL, SIL, TSIL, VAR, and VIN, the concentration range of 0.5–50.0% w/w with correlation scores ranging from 0.75 to 0.99 for FLU, MEL, and PEA, and the concentration range of 2.5–50.0% w/w with correlation scores ranging from 0.73 to 0.99 for PIP and SYN. The detectable concentration ranges as well as the Pearson's correlation scores of each adulterant obtained using the MISA Cal and R software are shown in Table 2.

3.5.2. Analysis of artificially adulterated DS samples

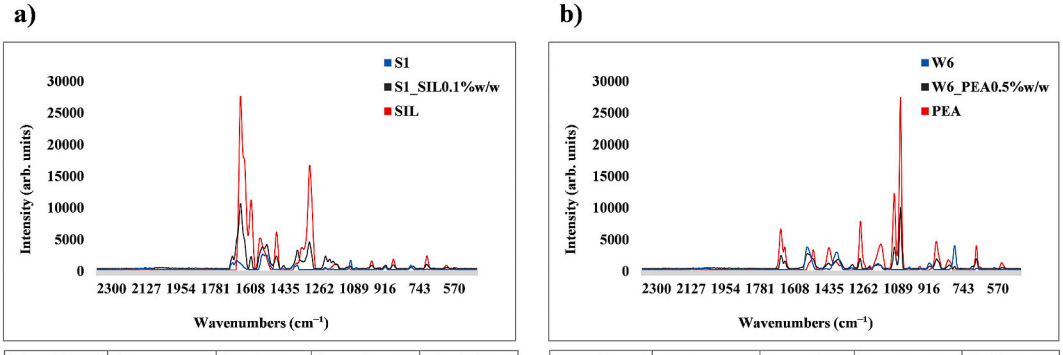
The last phase of this study involved the analysis of eighteen artificially adulterated commercial DS samples to evaluate the applicability of the developed SERS screening method to real conditions of adulteration and estimate the Limit of Identification (LOI) for the target adulterants. The LOI is calculated to estimate the lowest concentration of the analytes of interest for which correct identifications can be made from a defined database [49]; a library match score higher than 0.70 was established as identification criterion to estimate the LOI for each adulterant.

The blank commercial DS samples were first analyzed to confirm if they tested negative to the presence of the adulterants of interest and, therefore, to verify that the method does not provide false positive results. Then, the samples were artificially adulterated using the adulterants of interest, at low concentration levels (range 0.1–5.0% w/w), and the commercial DS samples as filling materials. Fig. 3 shows the SERS spectra of two blank DS (S1 and W6), their artificially adulterated forms with SIL and PEA respectively at the LOI levels (0.1 and 0.5% w/w), and the relative match scores with the library reference spectra of the two adulterants, which are also shown in the figure.

Under real conditions of adulteration, the developed screening method showed to be highly effective in detecting all the PDE5I tested (ACD, HSIL, SIL, TSIL, and VAR), with high match scores (0.79–0.99) even at the lowest concentration level tested (LOI of 0.1% w/w). This means that the developed screening method allows for the detection of some of the best known PDE5I and their unapproved analogues, added as adulterants in DS, below the concentration levels of the active therapeutic doses of the related pharmaceutical products on the market (Viagra® for sildenafil and Levitra® for vardenafil). Regarding the remaining SERS active analytes, the screening method showed to be capable of detecting VIN and PEA with a LOI of 0.5% w/w, PIP with a LOI of 2.5% w/w, and MEL, FLU, and SYN with a LOI of 5.0% w/w. The results are shown in Table S3.

4. Conclusions

This study presents the successful development of a multi-analyte screening method for rapid, in situ detection of illicit adulterants in DS. The aim of the study was to explore a new methodology to rapidly detect illegally added pharmaceutically active substances to dietary supplements. Surface-enhanced Raman spectroscopy (SERS) was selected as a promising methodology, due to its high sensitivity, rapidity, low cost, portability and suitability for in-situ sampling and monitoring. The method proved to be highly effective in detecting adulterations of the target PDE5I in sexual and sport performance enhancement supplements. Regarding the other SERS active adulterants covered by this study, it was not possible to detect them in all the tested samples at low concentration levels. To this regard, it should be specified that pharmacologically active adulterants are commonly added in relatively high concentrations to DS as the purpose is precisely to induce the desired effect in consumers; low concentrations would not be able to reach the minimum effective dose to produce the pharmacological effect.



Hazard Level	Name	Sample type	Source library	Match Score
Alarm	Sildenafil	Illicit Drug	Adulterants	0.82
Alarm	Acetildenafil	Illicit Drug	Adulterants	0.82
Alarm	Homo sildenafil	Illicit Drug	Adulterants	0.79

Hazard Level	Name	Sample type	Source library	Match Score
Alarm	Phenethylamine	Illicit Drug	Adulterants	0.81

Fig. 3. Surface-enhanced Raman spectroscopy (SERS) spectra of two blank dietary supplements and their artificially adulterated forms at the LOI levels, and library match scores with the two reference adulterants sildenafil (a) and phenethylamine (b).

The high selectivity of the silver *p*-SERS substrates for the tested adulterants, the non-existent interferences of the widely ranging composition of the supplements and the simplicity of the sample pre-treatment and analysis are valuable features of the methodology. Furthermore, the possibility of adding new SERS spectra of adulterants to the internal library of the software allows to re-interrogate a previously acquired sample to test its positivity to a new identified illicit adulterant. The developed method offers the possibility to quickly screen for specific adulterants in a capsule or tablet of a DS and it will be transferred to law enforcement, food safety authorities or customs' staff, in the near future.

Author contribution statement

Serena Rizzo: Conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; wrote the paper. **Yannick Weesepoel**: Conceived and designed the experiments; wrote the paper. **Sara Erasmus**: Conceived and designed the experiments; wrote the paper. **Joost Sinkeldam**: performed the experiments; contributed reagents, materials, analysis tools or data. **Anna Lisa Piccinelli**: Conceived and designed the experiments; wrote the paper. **Saskia van Ruth**: Conceived and designed the experiments; contributed reagents, materials, analysis tools or data; wrote the paper.

Data availability statement

Data associated with this study has been deposited at https://data.mendeley.com/datasets/y4md8znppn/1.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been publish online at [URL].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The analytical work was funded by the Ministry of Agriculture, Nature and Food Quality of the Netherlands through the WOT 02 Food Safety program of Wageningen Food Safety Research (project nr. WOT-02-005-044). The authors gratefully acknowledge technical support by Mr. Alex Koot, Ms. Vasiliki Akridopoulou, and Dr. Martin Alewijn of Wageningen Food Safety Research in the Netherlands.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18509.

References

[1] V.M. Wheatley, J. Spink, Defining the public health threat of dietary supplement fraud, Compr. Rev. Food Sci. Food Saf. 12 (2013) 599–613, https://doi.org/10.1111/1541-4337.12033.

[2] B. Egan, C. Hodgkins, R. Shepherd, L. Timotijevic, M. Raats, An overview of consumer attitudes and beliefs about plant food supplements, Food Funct. 2 (2011) 747, https://doi.org/10.1039/c1fo10109a.

[3] European Commision, Directive 2002/46/EC of the European parliament and of the Council of 10 june 2002 on the approximation of the laws of the member states relating to food supplements (text with EEA relevance), Official Journal of the European Communities 45 (2002) 51–57. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:L:2002:183:FULL&from=EN. accessed October 11, 2022.

[4] M. Avigan, R. Mozersky, L. Seeff, Scientific and regulatory perspectives in herbal and dietary supplement associated hepatotoxicity in the United States, Int. J. Mol. Sci. 17 (2016) 331, https://doi.org/10.3390/ijms17030331.

[5] J.W.H. Biesterbos, D.T.H.M. Sijm, R. van Dam, H.G.J. Mol, A health risk for consumers: the presence of adulterated food supplements in The Netherlands, Food Addit. Contam. 36 (2019) 1273–1288, https://doi.org/10.1080/19440049.2019.1633020.

[6] L. Manning, M. Bieniek, A. Kowalska, R. Ward, Dietary supplements, harm associated with synthetic adulterants and potential governance solutions, Crime Law Soc. Change (2021) 1–27, https://doi.org/10.1007/s10611-021-09992-9.

[7] E.H. Kim, H.S. Seo, N.Y. Ki, N.-H. Park, W. Lee, J.A. Do, S. Park, S.Y. Baek, B. Moon, H. Bin Oh, J. Hong, Reliable screening and confirmation of 156 multi-class illegal adulterants in dietary supplements based on extracted common ion chromatograms by ultra-high-performance liquid chromatography-quadrupole/time of flight-mass spectrometry, J. Chromatogr. A 1491 (2017) 43–56, https://doi.org/10.1016/j.chroma.2017.02.032.

[8] T. Rocha, J.S. Amaral, M.B.P.P. Oliveira, Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review, Compr. Rev. Food Sci. Food Saf. 15 (2016) 43–62, https://doi.org/10.1111/1541-4337.12173.

[9] N. Schramek, U. Wollein, W. Eisenreich, Identification of new synthetic PDE-5 inhibitors analogues found as minor components in a dietary supplement, J. Pharm. Biomed. Anal. 96 (2014) 45–53, https://doi.org/10.1016/j.jpba.2014.03.023.

[10] J. Yun, K.J. Shin, J. Choi, C.-H. Jo, Isolation and structural characterization of a novel sibutramine analogue, chlorosipentramine, in a slimming dietary supplement, by using HPLC-PDA, LC–Q-TOF/MS, FT-IR, and NMR, Forensic Sci. Int. 286 (2018) 199–207, https://doi.org/10.1016/j.forsciint.2018.03.021.

[11] J.H. Lee, H.N. Park, A. Jung, S. Mandava, S. Park, J. Lee, H. Kang, Isolation and characterisation of a novel sildenafil analogue adulterant, desmethylpiperazinyl propoxysildenafil, in a dietary supplement, Sci. Justice 58 (2018) 447–454, https://doi.org/10.1016/j.scijus.2018.07.003.

[12] Y. Gu, Q. Hu, J. Sun, H. Yu, H. Pan, X. Zhao, S. Ji, Isolation and identification of a new sildenafil analogue, hydroxycarbodenafil, found as an adulterant in a health supplement, J. Pharm. Biomed. Anal. 185 (2020), 113222, https://doi.org/10.1016/j.jpba.2020.113222.

[13] C. Li, X. Wang, S. Wu, J. Zhao, J. Fang, H. Li, Y. Zhu, K. Zhang, J. Peng, J. Mao, W. Li, K. He, N. Wang, F. Dong, Separation and structural elucidation of a novel vardenafil analogue as an adulterant in a natural health care dietary supplement, Heliyon 9 (2023), e15418, https://doi.org/10.1016/j.heliyon.2023.e15418.

[14] M.Z. Koncic, Getting more than you paid for: unauthorized “natural” substances in herbal food supplements on EU market, Planta Med. 84 (2018) 394–406, https://doi.org/10.1055/s-0044-100042.

[15] N. Basaran, D. Pasli, A.A. Basaran, Unpredictable adverse effects of herbal products, Food Chem. Toxicol. 159 (2022), 112762, https://doi.org/10.1016/j.fct.2021.112762.

[16] T. Ekar, S. Kreft, Common risks of adulterated and mislabeled herbal preparations, Food Chem. Toxicol. 123 (2019) 288–297, https://doi.org/10.1016/j.fct.2018.10.043.

[17] European Commission, Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, Official J. Europ. Commun. 45 (2002) 1–25. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:L:2002:031:FULL&from=EN. accessed October 12, 2022.

[18] European Commission, The rapid alert system for food and feed - annual report 2002. https://ec.europa.eu/food/system/files/2016-10/rasff_annual_report_2002_en.pdf, 2003 accessed October 12, 2022.

[19] European Commission, The rapid alert system for food and feed - annual report 2003. https://ec.europa.eu/food/system/files/2016-10/rasff_annual_report_2003_en.pdf, 2004 accessed October 12, 2022.

[20] European Commission, The rapid alert system for food and feed - annual report 2004, Luxembourg. https://ec.europa.eu/food/system/files/2016-10/rasff_annual_report_2004_en.pdf, 2005 accessed October 12, 2022.

[21] European Commission, The Rapid Alert System for Food and Feed - Annual Report 2005, Luxembourg, 2006.

[22] European Commission, The Rapid Alert System for Food and Feed - Annual Report 2006, Luxembourg, 2007.

[23] European Commission, The rapid alert system for food and feed - annual report 2007, Luxembourg. https://doi.org/10.2772/63727, 2008.

[24] European Commission, The rapid alert system for food and feed - annual report 2008, Luxembourg. https://doi.org/10.2772/72905, 2009.

[25] European Commission, The Rapid Alert System for Food and Feed - Annual Report 2009, Publications Office of the European Union, Luxembourg, 2010, https://doi.org/10.2772/88477.

[26] European Commission, The rapid alert system for food and feed - annual report 2010, Luxembourg. https://doi.org/10.2772/59932, 2011.

[27] European Commission, The rapid alert system for food and feed - annual report 2011, Luxembourg. https://doi.org/10.2772/96194, 2012.

[28] European Commission, The rapid alert system for food and feed - annual report 2012, publications office, Luxembourg. https://data.europa.eu/doi/10.2772/48887, 2013 accessed October 12, 2022.

[29] European Commission, The rapid alert system for food and feed - annual report 2013, Luxembourg. https://doi.org/10.2772/56447, 2014.

[30] European Commission, The rapid alert system for food and feed - annual report 2014, publications office, Luxembourg. https://doi.org/10.2875/945461, 2015.

[31] European Commission, The rapid alert system for food and feed - annual report 2015, Luxembourg. https://doi.org/10.2875/603169, 2016.

[32] European Commission, The rapid alert system for food and feed - annual report 2016, Luxembourg. https://data.europa.eu/doi/10.2875/022237, 2017.

[33] European Commission, The rapid alert system for food and feed - annual report 2017, Luxembourg. https://data.europa.eu/doi/10.2875/767865, 2018 accessed October 11, 2022.

[34] European Commission, The rapid alert system for food and feed - annual report 2018, Luxembourg. https://doi.org/10.2875/914558, 2019.

[35] European Commission, The rapid alert system for food and feed - annual report 2019, Luxembourg. https://data.europa.eu/doi/10.2875/993888, 2020 accessed October 11, 2022.

[36] European Commission, The rapid alert system for food and feed - annual report 2020, Luxembourg. https://doi.org/10.2875/259374, 2021.

[37] L. Muschietti, F. Redko, J. Ulloa, Adulterants in selected dietary supplements and their detection methods, Drug Test. Anal. 12 (2020) 861–886, https://doi.org/10.1002/dta.2806.

[38] M. Yelehe-Okouma, E. Pape, L. Humbertjean, M. Evrard, R. El Osta, N. Petitpain, P. Gillet, S. El Balkhi, J. Scala-Bertola, Drug adulteration of sexual enhancement supplements: a worldwide insidious public health threat, Fundam. Clin. Pharmacol. 35 (2021) 792–807, https://doi.org/10.1111/fcp.12653.

[39] M. Petersen, Z. Yu, X. Lu, Application of Raman spectroscopic methods in food safety: a review, Biosensors 11 (2021) 187, https://doi.org/10.3390/bios11060187.

[40] J. Langer, D. Jimenez de Aberasturi, J. Aizpurua, R.A. Alvarez-Puebla, B. Auguie, J.J. Baumberg, G.C. Bazan, S.E.J. Bell, A. Boisen, A.G. Brolo, J. Choo, D. Cialla-May, V. Deckert, L. Fabris, K. Faulds, F.J. Garcia de Abajo, R. Goodacre, D. Graham, A.J. Haes, C.L. Haynes, C. Huck, T. Itoh, M. Kall, J. Kneipp, N.A. Kotov, H. Kuang, E.C. Le Ru, H.K. Lee, J.-F. Li, X.Y. Ling, S.A. Maier, T. Mayerhofer, M. Moskovits, K. Murakoshi, J.-M. Nam, S. Nie, Y. Ozaki, I. Pastoriza-Santos, J. Perez-Juste, J. Popp, A. Pucci, S. Reich, B. Ren, G.C. Schatz, T. Shegai, S. Schlucker, L.-L. Tay, K.G. Thomas, Z.-Q. Tian, R.P. Van Duyne, T. Vo-Dinh, Y. Wang, K.A. Willets, C. Xu, H. Xu, Y. Xu, Y.S. Yamamoto, B. Zhao, L.M. Liz-Marzan, Present and future of surface-enhanced Raman scattering, ACS Nano 14 (2020) 28–117, https://doi.org/10.1021/acsnano.9b04224.

[41] Y. Yang, N. Creedon, A. O’Riordan, P. Lovera, Surface enhanced Raman spectroscopy: applications in agriculture and food safety, Photonics 8 (2021) 568, https://doi.org/10.3390/photonics8120568.

[42] M. de Veij, P. Vandenabeele, T. De Beer, J.P. Remon, L. Moens, Reference database of Raman spectra of pharmaceutical excipients, J. Raman Spectrosc. 40 (2009) 297–307, https://doi.org/10.1002/jrs.2125.

[43] P. Mosier-Boss, Review of SERS substrates for chemical sensing, Nanomaterials 7 (2017) 142, https://doi.org/10.3390/nano7060142.

[44] E.P. Hoppmann, W.W. Yu, I.M. White, Highly sensitive and flexible inkjet printed SERS sensors on paper, Methods 63 (2013) 219–224, https://doi.org/10.1016/j.ymeth.2013.07.010.

[45] F. Fang, Y. Qi, F. Lu, L. Yang, Highly sensitive on-site detection of drugs adulterated in botanical dietary supplements using thin layer chromatography combined with dynamic surface enhanced Raman spectroscopy, Talanta 146 (2016) 351–357, https://doi.org/10.1016/j.talanta.2015.08.067.

[46] H. Li, Q. xia Zhu, T. sian Chwee, L. Wu, Y. feng Chai, F. Lu, Y. fang Yuan, Detection of structurally similar adulterants in botanical dietary supplements by thin-layer chromatography and surface enhanced Raman spectroscopy combined with two-dimensional correlation spectroscopy, Anal. Chim. Acta 883 (2015) 22–31, https://doi.org/10.1016/j.aca.2015.04.017.

[47] Q. Zhu, Y. Cao, Y. Cao, Y. Chai, F. Lu, Rapid on-site TLC–SERS detection of four antidiabetes drugs used as adulterants in botanical dietary supplements, Anal. Bioanal. Chem. 406 (2014) 1877–1884, https://doi.org/10.1007/s00216-013-7605-7.

[48] H. Alyami, E. Dahmash, J. Bowen, A.R. Mohammed, An investigation into the effects of excipient particle size, blending techniques and processing parameters on the homogeneity and content uniformity of a blend containing low-dose model drug, PLoS One 12 (2017), e0178772, https://doi.org/10.1371/journal.pone.0178772.

[49] E. Massarini, P. Wasterby, L. Landstrom, C. Lejon, O. Beck, P.O. Andersson, Methodologies for assessment of limit of detection and limit of identification using surface-enhanced Raman spectroscopy, Sensor. Actuator. B Chem. 207 (2015) 437–446, https://doi.org/10.1016/j.snb.2014.09.116.