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RESEARCH ARTICLE

A simple method for preparing herbal reference standards based on salting-out solvent extraction

Igor Casian*, Ana Casian

Centre for Drug Development, *Nicolae Testemițanu* State University of Medicine and Pharmacy, Chișinău, Republic of Moldova

ABSTRACT

Introduction. The expansion of the range of herbal medicinal products requires the availability of a large number of different reference standards for their analysis. A possible solution is the use of herbal reference standards (HRS), which, in turn, requires the development of simple production methods that meet all the requirements for reference standards.

Material and methods. Using ten plant species containing polyphenolic compounds, a general scheme for preparing HRS was developed and tested, which includes extraction of plant material with low concentrations of a polar organic solvent (usually 20% isopropanol), subsequent salting-out of the organic phase with ammonium sulfate, and drying the organic extract on the surface of anhydrous lactose. The composition of the obtained HRS and intermediates was determined by high-performance liquid chromatography using primary chemical reference standards.

Results. For all studied plant species, satisfactory values of the yield of target components, good solubility of the prepared HRS, and similarity of the chromatographic profiles of HRS and the corresponding plant material were obtained.

Conclusions. A simple and inexpensive method for preparing HRS, based on salting-out solvent extraction of target components, is proposed.

Keywords: herbal reference standards, salting-out solvent extraction, polyphenolic compounds, phytochemical analysis, pharmaceutical analysis.

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Corresponding author: Igor Casian, MD, PhD, Research Associate Professor
Centre for Drug Development
Nicolae Testemițanu State University of Medicine and Pharmacy
Malina Mica str. 66, Chișinău, Republic of Moldova, MD-2025
e-mail: igor.casian@usmf.md

Authors' ORCID IDs
Igor Casian – https://orcid.org/0000-0001-6392-3804
Ana Casian – https://orcid.org/0000-0001-8876-3691

Key messages

What is not yet known on the issue addressed in the submitted manuscript

The possibility of obtaining herbal reference standards (HRS) in the form of dry extracts using the same solvent for treating the plant material followed by salting-out extraction of target compounds.

The research hypothesis

Preparing the primary plant extract with an organic solvent that can be salted out in a subsequent technological stage should reduce time and solvent consumption, thereby simplifying the entire HRS preparation process.

The novelty added by manuscript to the already published scientific literature

Using the same water-organic mixture for preparing the primary plant extract and for subsequent salting-out extraction showed a good yield of medium-polar compounds, such as polyphenols, resulting in a very simple and inexpensive method for producing HRS.

Introduction

The constant expansion of the range of medicinal and dietary products of plant origin, as well as the increasing requirements for their quality, has led to the wide use of instrumental (especially chromatographic) methods of analysis. The multicomponent composition of plant materials poses a serious problem associated with the need to use a significant number of reference chemical substances for calibration and selectivity testing, many of which are expensive or difficult to access.

The European Pharmacopoeia and other international pharmacopoeias propose the use of herbal reference standards (HRS) as a solution to this problem [1-4]. HRS are herbal materials or extracts, usually dry, intended, in most cases, for use as primary standards for qualitative analysis (identification of chromatographic peaks and/or system suitability testing). Pharmacopoeias do not recommend, but do not exclude, the use of HRS in quantitative analysis. In this case, HRS are considered secondary standards and should be standardized against primary chemical standards. In the absence of appropriate pharmacopeial reference standards, non-pharmacopoeial reference standards that meet the requirements of Ph. Eur., chapter 5.12 "Reference standards", are developed and implemented. At the stage of extensive studies carried out during the development of new pharmaceutical products, appropriately characterized primary and working in-house reference materials are used [5].

Factors limiting the use of HRS for quantitative analysis are the hygroscopicity of dry extracts, which imposes high requirements on packaging and storage conditions, solubility problems associated with matrix components, and the uncertainty of chromatogram processing results caused by peak interference and matrix effects [6]. A solution to the problem of HRS hygroscopicity and solubility may be the removal of both the most polar and least polar groups of matrix components at the stage of primary extract purification [7].

The salting-out liquid extraction (also known as homogeneous liquid extraction) method is based on the separation of a mixture of solvents miscible under normal conditions (usually water and a polar organic solvent) by the addition of strong electrolytes. It has been used by various authors for the extraction of metal ions from aqueous solutions [8], determination of preservatives and sweeteners in juices [9], α-dicarbonyl compounds in beer [10], iodate in food-grade salt [11], in biomedical analysis [12, 13], and many other cases. It was previously used to obtain HRS from such plant species as *Melissa officinalis* L., *Hypericum perforatum* L., *Crataegus monogyna* Jacq., *Urtica dioica* L., and *Sambucus nigra* L., showing good efficiency in removing highly polar ballast components [7]. In this case, salting-out extraction with isopropanol in the presence of ammonium sulfate was used after the distillation of ethanol from the primary water-alcoholic extract, which increased the labor intensity and energy consumption of the entire technological process and required the use of two different organic solvents.

In this article, the possibility of salting-out liquid extraction of target components directly from the primary plant extract, without prior removal of the organic solvent, and the efficiency of extraction of various groups of polyphenolic compounds using this approach are discussed.

The aim of the study is to develop the most efficient and cost-effective method for preparing HRS, based on salting-out liquid extraction.

Material and methods

Plant material. Wild bergamot (*Monarda fistulosa* L.), Melissa (*Melissa officinalis* L.), Tansy (*Tanacetum vulgare* L.), and St. John's wort (*Hypericum perforatum* L.) were harvested from the plantation of the Scientific-Practical Centre in the Domain of Medicinal Plants of *Nicolae Testemițanu* State University of Medicine and Pharmacy; Nettle (*Urtica dioica* L.), Hawthorn (*Crataegus monogyna* Jacq.), and Elder flower (*Sambucus nigra* L.) – from the spontaneous flora (municipality Chișinău, township Codru). Collected aerial parts or appropriate organs of the studied plants were air-dried and fragmented to a particle size of 0.65-1.4 mm. Green tea leaves (*Camellia sinensis* (L.) Kuntze) according to the technical specification TU U 19421419.001-99 ("Monomakh" JSC, Ukraine), fresh orange (*Citrus* × *sinensis* (L.) Osbeck) according to the international standard CODEX STAN 245-2004 (Spain), and grapefruit (*Citrus paradisi* Macfad.) according to the international standard CODEX STAN 219-1999 (South Africa) were purchased from a local grocery store.

Apparatus. The plant material was extracted in a cylindrical flow extractor with a 50 mL internal volume, using a peristaltic pump for solvent delivery. Analysis of plant material, extracts, and the final preparations was performed using "Agilent 1260 Infinity" liquid chromatograph with a diode-array detector.

Chemicals. Primary reference substances, solvents, and reagents of analytical grade used in the study were purchased from Sigma-Aldrich (USA), Merck, Fluka, and Stanchem (Germany).

Methodology. The following general scheme was elaborated and used for preparing HRS:

10-20 g of dried and fragmented plant material (depending on its bulk density and swelling coefficient) was loaded into a flow extractor. A 20% isopropanol or another selected solvent was fed into the bottom of the extractor at room or controlled temperature and at a flow rate of 15-25 mL/h using a peristaltic pump, until 2.5–3 parts (*V/M*) of the primary extract were obtained. After taking an analytical sample, ammonium sulfate was dissolved in the extract to saturation (approximately 0.45-0.5 grams per milliliter of primary extract at room temperature). The mixture was centrifuged for 5 min at 3000 min^{-1}, the upper liquid layer was separated, its volume was measured, and a sample was taken for analysis. The organic extract was then applied in 2 stages to a 2-fold (*M/V*) amount of anhydrous lactose with intermediate drying in an open porcelain cup at 70°C and continuous stirring. Final drying was performed for 16-20 h at 40°C. The target product was then lightly ground and sieved (0.65 mm mesh size).

In the case of HRS preparation from fruit juices, 40 mL of freshly squeezed juice was mixed with 10 mL of isopropanol, heated to 80°C to coagulate protein substances, cooled to +4°C, and centrifuged. The centrifugate was saturated with ammonium sulfate, the phases were separated by centrifugation, and the upper layer was applied in 3 stages to 1.5-fold (*M/V*) amount of anhydrous lactose and dried as described above.

The HRS obtained by the described method were dissolved for analysis in 20% ethanol (about 100 mg per 10 mL), visually assessing the completeness of dissolution. The chromatographic profiles of HRS and the original plant material were compared using the "fingerprint method". The quantitative content of the identified components was also determined using primary reference substances.

Results

For all plant species included in the study, good similarity of the chromatographic profiles of HRS and the corresponding raw materials was achieved, which allows the obtained HRS to be considered suitable for peak identification and testing of chromatographic system selectivity. All prepared HRS were completely soluble in 20% ethanol, except Wild bergamot HRS, which needed to be dissolved in 1 mL of dimethyl sulfoxide before dilution with the main solvent, due to the presence of flavone glycosides poorly soluble in water-alcohol mixtures.

The composition of the extractants used, the extraction temperature, as well as the content of the identified components and their yield from the initial plant material, are given in Table 1.

Table 1. Basic conditions for the extraction of plant material and polyphenols composition of the obtained HRS and intermediate products

Plant material	Extractant and temperature	Quantified compounds	Concentration (yield from plant material)		
			Primary extract	Salted-out extract	HRS
Wild bergamot herb	20% IPA, 20-22°C	Rosmarinic acid	1.98 mg/mL (57.7%)	9.69 mg/mL (56.4%)	4.19 mg/g (52.5%)
		Monardic acid A as salvianolic acid A	3.77 mg/mL (71.6%)	12.3 mg/mL (46.7%)	5.29 mg/g (43.2%)
		Flavone glycosides as cynaroside	7.34 mg/mL (77.3%)	15.7 mg/mL (33.1%)	6.99 mg/g (31.7%)
		Flavanone glycosides as naringin	1.56 mg/mL (67.4%)	6.29 mg/mL (54.4%)	2.75 mg/g (51.1%)
Melissa leaf	20% IPA, 20-22°C	Rosmarinic acid	7.32 mg/mL (74.7%)	33.1 mg/mL (71.3%)	13.6 mg/g (64.9%)
Tansy flower	20% IPA, 20-22°C	Hydroxycinnamic acids as chlorogenic acid	9.00 mg/mL (52.4%)	42.0 mg/mL (51.4%)	17.7 mg/g (46.9%)
Nettle leaf	20% IPA, 20-22°C, then + H_3PO_4 to pH 3.0	Chlorogenic acid	2.49 mg/mL (92.7%)	12.6 mg/mL (86.3%)	5.25 mg/g (77.1%)
		Cafeoilmalic acid as chlorogenic acid	4.61 mg/mL (95.3%)	23.8 mg/mL (92.6%)	10.4 mg/g (86.7%)
Hawthorn leaf and flower	20% IPA, 20-22°C	Hydroxycinnamic acids as chlorogenic acid	3.42 mg/mL (73.3%)	11.8 mg/mL (42.1%)	5.05 mg/g (38.9%)
		Flavonol glycosides as hyperoside	0.93 mg/mL (62.8%)	4.07 mg/mL (45.6%)	1.74 mg/g (41.0%)
		Flavone glycosides as vitexin-2-O-rhamnoside	3.73 mg/mL (69.5%)	16.1 mg/mL (50.0%)	6.92 mg/g (45.1%)
Elder flower	15% ACN + 10% IPA, 50°C	Hydroxycinnamic acids as chlorogenic acid	3.71 mg/mL (65.3%)	11.3 mg/mL (50.5%)	5.27 mg/g (49.7%)
		Flavonol glycosides as rutoside	3.82 mg/mL (68.5%)	11.9 mg/mL (54.0%)	5.42 mg/g (52.2%)
St. John's wort	15% ACN + 10% IPA, 45°C	Hydroxycinnamic acids as chlorogenic acid	1.15 mg/mL (55.6%)	3.87 mg/mL (44.2%)	1.53 mg/g (37.5%)
		Flavonol glycosides as rutoside	0.54 mg/mL (49.8%)	2.20 mg/mL (41.0%)	0.84 mg/g (34.9%)
		Hypericin + pseudohypericin	0.28 mg/mL (23.8%)	1.12 mg/mL (22.7%)	0.44 mg/g (19.2%)
Green tea leaf	20% IPA, 20-22°C	Caffeine	5.05 mg/mL (78.9%)	22.3 mg/mL (76.6%)	8.92 mg/g (67.6%)
		Total catechins as (-)-epicatechin	36.2 mg/mL (74.4%)	154 mg/mL (69.6%)	60.0 mg/g (59.6%)
Orange juice	IPA, 25% from juice volume	Hesperidin	–	1.20 mg/mL (53.8%)	0.80 mg/g (52.6%)
Grapefruit juice	IPA, 25% from juice volume	Naringin	–	5.09 mg/mL (77.0%)	3.10 mg/g (73.6%)

Note: HRS – herbal reference standard; IPA – isopropanol; ACN – acetonitrile.

A special study on the efficiency of salting-out agents was carried out using Wild bergamot extracts, containing various groups of polyphenolic compounds, as an example (Table 2).

Table 2. The efficiency of salting-out extraction from 20% isopropanol using saturating concentrations of various salts (for some polyphenolic compounds of Wild bergamot)

Salting-out agent	Analyte concentration (yield from the primary extract)			
	Rosmarinic acid	Monardic acid A	Flavone glycosides	Flavanone glycosides
Ammonium sulfate	8.63 mg/mL (100%)	10.7 mg/mL (73%)	15.5 mg/mL (51%)	5.84 mg/mL (95%)
Magnesium sulfate	6.23 mg/mL (96%)	9.24 mg/mL (85%)	19.8 mg/mL (78%)	4.63 mg/mL (100%)
Sodium sulfate	5.77 mg/mL (100%)	8.43 mg/mL (87%)	16.1 mg/mL (80%)	3.79 mg/mL (92%)
Sodium thiosulfate	7.62 mg/mL (77%)	6.45 mg/mL (39%)	10.8 mg/mL (31%)	5.63 mg/mL (80%)
Sodium citrate	6.02 mg/mL (44%)	5.25 mg/mL (23%)	12.9 mg/mL (27%)	4.86 mg/mL (49%)
Sodium nitrate	3.87 mg/mL (42%)	2.06 mg/mL (13%)	4.75 mg/mL (15%)	3.00 mg/mL (45%)
Sodium nitrite	1.09 mg/mL (7.9%)	0.25 mg/mL (1.1%)	2.41 mg/mL (5.0%)	2.86 mg/mL (29%)
Sodium chloride	3.38 mg/mL (29%)	1.39 mg/mL (7.1%)	3.67 mg/mL (9.1%)	2.90 mg/mL (35%)
Sodium acetate	n/s	n/s	n/s	n/s
Ammonium acetate	n/s	n/s	n/s	n/s
Ammonium chloride	n/s	n/s	n/s	n/s
Potassium chloride	n/s	n/s	n/s	n/s
Potassium bromide	n/s	n/s	n/s	n/s
Potassium iodide	n/s	n/s	n/s	n/s
Potassium dihydrogen phosphate	n/s	n/s	n/s	n/s
Potassium oxalate	n/s	n/s	n/s	n/s

Note: n/s – no separation of liquid phases.

Discussion

Isopropanol was used as the organic component of the extraction mixture for most plant materials because it is sufficiently polar and can be easily salted out from aqueous solutions. Previous studies [7] showed good extraction of a wide range of polyphenolic compounds into the organic phase using the salting-out method. At concentrations of about 20%, it provides a sufficiently high yield of most polyphenolic compounds during the extraction of plant material while minimizing the extraction of undesirable low-polar components, such as lipids and resins. Removal of the most polar ballast (carbohydrates, hydroxy acids, inorganic salts) is ensured at the subsequent stage of salting out the organic phase. The availability and relatively low toxicity of isopropanol are also attractive properties.

However, in some cases, we observed insufficient extraction of all or individual target components from the plant material. For Elder flower at room temperature, an abnormally low (15-20%) yield of both chemical groups of polyphenols was obtained due to the high activity of polyphenol oxidase. In St. John's wort, we observed a significant conversion of quercetin glycosides into the aglycone due to hydrolase activity. In both cases, the problem was solved by adding acetonitrile (a protein-denaturing agent) to the extractant and increasing the extraction temperature. Complete replacement of isopropanol with acetonitrile led to a low yield of the most polar components; therefore, a small amount of isopropanol was retained in the extractant.

Salting out of the organic solvent and target components in all cases was performed with ammonium sulfate, as used by other authors [9, 11, 12]. The high water solubility of this salt allows minimizing the time required for the salting-out procedure and obtaining extracts with a high yield of target components and minimal water content. This circumstance facilitates the subsequent drying of the organic extracts. The advantage of this salting-out agent is demonstrated in Table 2, which shows that the highest yield of all components is provided by salts of polybasic acids, especially those of sulfuric acid. At the same time, ammonium sulfate gave the maximal concentration of most analytes due to the lowest water extraction.

When the primary water-organic extract is saturated with ammonium sulfate and centrifuged, the mixture usually separates into three liquid phases. The upper layer is an organic extract containing the target components; the lower layer is a concentrated aqueous solution of ammonium sulfate; and between them lies a resinous layer of small volume, representing a concentrated aqueous solution of highly polar organic compounds, such as mono- and oligosaccharides, with solid particles of polysaccharides, proteins, and other substances that are poorly soluble in organic solvents and concentrated electrolyte solutions. A solid sediment, consisting of excess ammonium sulfate and poorly soluble inorganic salts, such as calcium sulfate, may be present at the bottom of the centrifuge tube.

In most cases, primary plant extracts are naturally slightly acidic. An exception in this study was Nettle extracts, with pH values of 7.3-7.5, which resulted in a low yield of hydroxycinnamic acids, especially caffeoylmalic acid, upon salting out. In this case, o-phosphoric acid was used to adjust the pH.

In general, the obtained results confirm the suitability of the proposed method for the production of HRS, at least from polyphenol-containing plants. The method is time-saving (all operations, except the final drying, were performed within one working day) and material-saving,

both in relation to plant raw materials and to solvents and reagents. This allows its wide use in research and educational practice. The obtained HRS were found to be the most convenient as in-laboratory working standards for the study of non-pharmacopeial plant species, their screening, phenotyping, technological, and other studies associated with large volumes of analytical work, allowing for significant savings of expensive chemical reference standards.

The use of fresh plant juices eliminates the stage of obtaining the primary extract and simplifies the entire technological process. However, the target substances must pass into the squeezed juice in sufficient amounts. Most likely, HRS prepared from fresh juices will be suitable for the analysis of the juices themselves, particularly in the food industry. Thus, the HRS samples obtained in this study from grapefruit and orange juices may be useful for identifying cases of adulteration of grapefruit juice by the presence of hesperidin.

Conclusions

Extraction of plant material with a 20% isopropanol solution, followed by salting out the organic solvent with ammonium sulfate, in most cases showed a sufficiently high and uniform yield of various groups of polyphenolic compounds. Subsequent drying of the organic extracts on the surface of anhydrous lactose allowed the obtaining of non-hygroscopic, easily soluble dry extracts suitable for qualitative (as primary standards) or quantitative (as secondary standards) analysis of the corresponding plant materials and preparations. As a result, a simple and inexpensive method for preparing herbal reference standards using salting-out liquid extraction was proposed.

Competing interests

None declared.

Authors' contributions

IC designed the study, conducted the laboratory work and performed its technological part, interpreted the data, and drafted the first manuscript. AC collected and processed the plant material, performed the analytical part of the laboratory work, and revised the manuscript. The final version of the manuscript was approved by all authors.

Ethics approval

Not needed for this study

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