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Development of a new maceration technique to optimize extraction and isolation of natural products

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ABSTRACT:

The extraction of active components from plants is the first crucial step in the field of natural product research. In the case of non-targeted extraction with an objective to isolate and characterize as many compounds as possible, the most classical technique, and the simplest to implement, is the Soxhlet extraction (SE). However, this method does not allow to extract all the compounds from the plant. Indeed, the use of several solvents allowing a variation of polarity is especially suitable since natural products are so diverse with various physicochemical properties (e.g. polarity, solubility...). Thus the second most used technique is the extraction by successive macerations using solvents of increasing polarity. If this method is frequently used, very few studies are available to rationalize and optimize it. Furthermore, this extraction technique still requires some enhancement mainly for efficiency, economic, environmental and time constraint reasons. In this work, we present an innovative method of successive macerations using a mixture of solvents with the aim of simultaneously improving the yield, the partition of the compounds between the different phases and reducing the volume of solvent. Triphasic systems were prepared by mixing five different solvents (*n*-heptane, ethyl acetate, acetonitrile, butan-1-ol and water) in various proportions. Seven natural compounds of different polarity were then dissolved in these mixtures. After decantation, an apolar, an intermediate and a polar phase were obtained and analyzed to evaluate the partition of each compound. The best triphasic system showed a very performant phase partition. Indeed, the majority of the relevant secondary metabolites are found in the intermediate phase while primary metabolites were recovered in apolar and polar phases. This triphasic system was subsequently used to perform three successive macerations with a polarity gradient on aerial parts of a model plant, *Anthyllis vulneraria* L. Our results showed an overall good yield compared to conventional maceration techniques, while reducing time of extractions and volume of solvents.

Keywords:

Natural products, extraction, maceration optimization, phase partition, triphasic solvent system

1 Introduction

Plant-derived substances are a valuable source of discovery of original chemical entities and of inspiration for the development of new bioactive compounds. For instance, regarding cancer drugs, Newman and Cragg have shown that about half of the drugs approved by the FDA between 1981 and 2010 are natural or derived from natural products.[1] The benefits brought by bioactive plant constituents and the positive impacts for consumers are even more important than conventional drugs which largely explains the fad of pharmaceutical, cosmeceutical, nutraceutical or food industries for developing and formulating natural source based products.

46 The first key steps in the development of bioactive chemical constituents from biomass are the pre-
47 extraction and the extraction procedures.[2] The technical choices made during these initial stages
48 must enable the biomolecules present in the plants to be preserved. Regarding the pre-extraction
49 steps, the more common practice is to leave the sample to dry at ambient temperature (or on an oven
50 at low temperature) in a ventilated place. This option is generally consistent with the time needed for
51 experimental design but should be reconsidered if volatile or thermolabile compounds are
52 requested.[3] The grinding step is of particular importance to increase the surface contact between
53 samples and solvents: it is widely estimated that a particle size smaller than 0.5 mm is ideal for
54 efficient extraction.[4] Afterwards, the quality of the isolated extract relies largely on the extraction
55 technique employed and can be evaluated by the mass percentage of recovered compounds. The
56 selection of the extraction method will be guided by various factors, for instance, the sample size, the
57 knowledge of the chemical family to isolate, their stability/sensitivity.[2,3,5,6,7] Conventional methods
58 such as maceration, infusion, percolation, decoction and Soxhlet extraction (SE) or hot continuous
59 extraction are still commonly used since they only require basic glassware and are convenient for both
60 small and large scale extraction. However, maceration and percolation are time and solvent-
61 consuming and the other methods are not suitable for thermosensitive compounds.[4,8] Recently
62 developed extraction methods, such as microwave-assisted extraction (MAE), ultrasound-assisted
63 extraction (UAE), supercritical-fluid extraction (SFE) or accelerated solvent extraction (ASE), offer
64 various advantages including to shorten the extraction time while achieving usually higher yield, to
65 reduce organic solvent consumption or to create selective recovery. Unfortunately, each method
66 suffers from its own limitations: 1) MAE is mainly limited to small polar molecules and generally not
67 suitable for thermosensitive compounds; 2) UAE is rarely applied to large-scale extraction and may
68 cause formation of free radicals affecting active compounds; 3) SFE and ASE are less frequently used
69 owing to the initial high cost of equipment. Consequently, unless one targets a special class of
70 metabolites requiring an optimization of the method, exhaustive or non-targeted extraction still involves
71 maceration as a very convenient method consuming little energy and with no detrimental effect on
72 thermosensitive compounds.[9,10,11,12,13] It has incidentally been recommended as the best
73 extraction option especially for small and medium enterprises and in developing countries.[9]

74 While maceration is nowadays widely used by phytochemists, research groups both in public and
75 private sectors usually perform extraction protocol based on in-house knowledge rather than on
76 validated experiments. Since metabolites present diverse physicochemical properties, notably polarity
77 and thus solubility, teams working in the field of natural products generally carry out successive
78 extractions with solvents of different polarity starting from apolar alkane-based solvents (e. g. heptan,
79 hexan, cyclohexan) to intermediate solvents (e. g. dichloromethan, ethyl acetate) and then to polar
80 solvents (alcohols and water).[14,15,16,17] This methodology will be referred as to “conventional
81 method” within this document. Despite its practical advantages and wide use, this conventional
82 method suffers from several drawbacks including a time- and solvent-consuming process and a low
83 extraction level of poorly soluble metabolites at room temperature. Moreover, a complete separation of
84 the natural constituents can rarely be achieved, and the same compounds may be recovered (in
85 varying proportions) within several fractions.

86 From a theoretical perspective, the perfect extraction technique should combine ease of use,
87 speedness, low-cost and a large-scale applicability. Our approach to improve the classical maceration
88 process is thus the development of a new triphasic solvent system that could be an effective method
89 to address the efficiency, economic, environmental and time constraint issues while promoting a better
90 partition of molecules in each phase. After preliminary tests to fine-tune our solvent mixture, the
91 performance of our new methodology has been evaluated by comparison with “conventional method”
92 using successive extraction by five solvents of increasing polarity. Thereafter, this methodology will be
93 referred as to “mixed method”.

94

95

96 **2 Materials and methods**

97

98 **2.1 Solvents and chemicals**

99 All solvents used for the preparation and analyses were of analytical grade and purchased from VWR
100 SAS. Chemical products (i.e. glucose, gallic acid, caffeic acid, rutin, rosmarinic acid, quercetin, aescin
101 and palmitic acid) were purchased from Sigma-Aldrich, Extrasynthese or Phytolab GmbH.

102

103 **2.2 HPLC and NMR analyses**

104 HPLC analyses were performed with an Agilent 1290 infinity system (Agilent Technologies) using
105 ELSD and DAD (280 nm) detectors, an Agilent Zorbax Eclipse Plus C18 RRHD (2.1 x 50 mm, 1.8 µm)
106 as stationary phase, with a mobile phase composed of A = H₂O and 0.1% formic acid and B =
107 methanol (MeOH) with a gradient from 95:5 to 10:90, over 17 min, 0.5 mL/min, column temperature:
108 50 °C, sample volume injection: 1 µL.

109 ¹H NMR spectra were recorded at 400 MHz and at room temperature in deuterated solvent (CDCl₃ or
110 DMSO-*d*₆) on a Brüker Avance-400 instrument.

111

112 **2.3 Plant collection and sample preparation**

113 The model plant used for this study, *A. vulneraria*, was harvested in the French Alps (Isola 2000, 2150
114 m) in accordance with good practice for plant collection and international treaties on biodiversity.
115 Plants were placed in an air-dry oven at 40 °C for 24 h or until no weight loss was observed. Then
116 they were grounded to powder using Retsch Grindomix GM 200.

117

118 **2.4 Extraction**

119 **2.4.1. Development of the triphasic system**

120 10 mL of each mixture of four or five solvents (*n*-heptan, EtOAc, acetonitrile, water and eventually
121 butan-1-ol) were thoroughly shaken to obtain, after decantation, a triphasic system. 20 mg of a mixture
122 in equal parts by weight of glucose, gallic acid, caffeic acid, rutin, rosmarinic acid, quercetin, palmitic
123 acid and eventually saponins was added to the solvents. After efficient stirring and decantation, the
124 composition of each phase was analyzed by HPLC-ELSD.

125 **2.4.2. Conventional method and/or mixed method**

126 The powdered plant material from *A. vulneraria* (50 g) was placed in a 1 L round bottom flask. The
127 appropriate volume of the more apolar solvent mixture was first added and the mixtures were stirred at
128 35 °C during 24 h. Every hour during 8 h and then at 24 h, a sample was collected to determine, using
129 HPLC-ELSD, the tracer compound concentration and saturation in each solvent. After 24 h, the
130 mixtures were filtered up to 0.4 µm. The plant residues were extracted again with the successive
131 solvents of increasing polarities. Each filtrate was evaporated and weighed to determine the yield.

132 **2.4.3. Soxhlet extraction procedure**

133 A cellulose cartridge was filled with 50.4 g of the powdered plant material from *A. vulneraria* and the
134 SE was carried out for 24 h using 1 L of MeOH. The mixture was filtered up to 0.4 µm, then
135 evaporated and weighed to determine the yield.

136

137

138

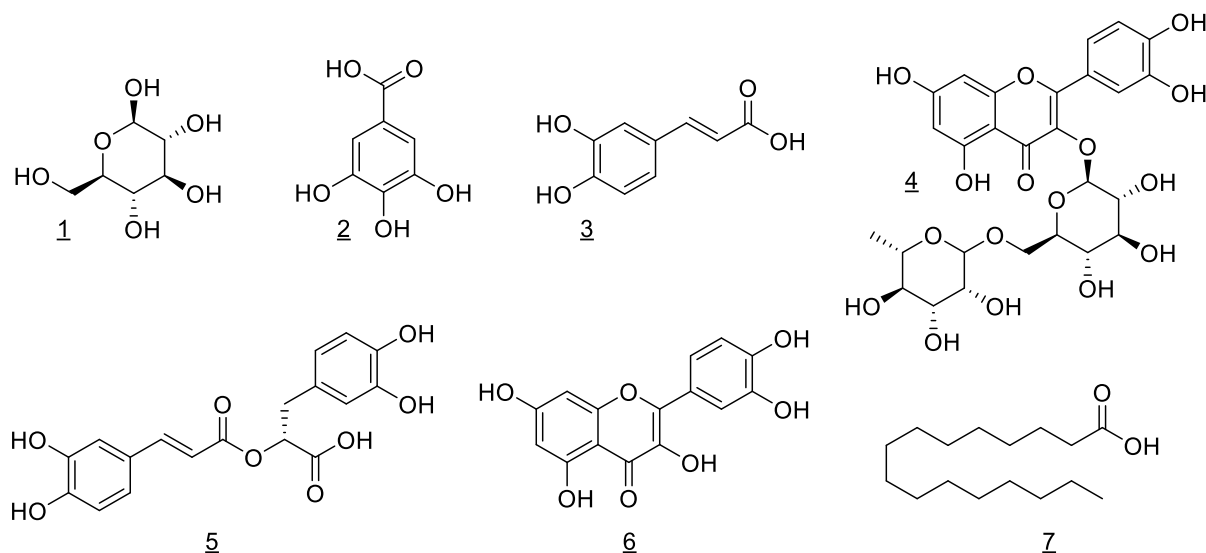
139 **3. Results and discussion**

140 To overcome issues related to maceration process, many efforts have been therefore devoted in this
141 study to improve the yield of extraction while reducing the time of experiment. In this context, a five
142 solvent mixture namely *n*-heptan, acetonitrile, butan-1-ol, ethyl acetate (EtOAc) and water, has been
143 developed and optimized to perform the successive extraction. The solvent mixture with varying
144 solvent ratios was used to produce a triphasic system, each phase subsequently used as a solvent for
145 the extraction. This original “mixed method” was evaluated by comparison to the “conventional
146 method” on the basis of yields.

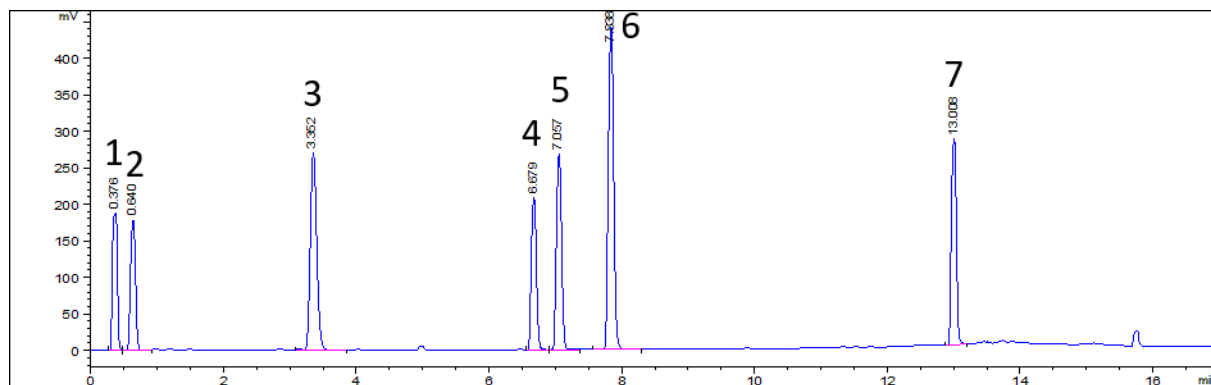
147

148 **3.1 Development of a new triphasic solvent system**

149 Selection of the organic solvents of different polarity used during the extraction process is fundamental
150 to ensure the solubility and then the extraction of the broad diversity of metabolites present in plants.
151 To conform to time and environmental constraints, our maceration methodology relies on only three
152 extraction phases: an apolar, an intermediate and a polar phase. The preliminary development of this
153 new triphasic system was based on the assessment of its efficiency to partition seven molecules of
154 different polarities, frequently found in plant extracts. This designed mixture is composed of glucose **1**,
155 gallic acid **2**, caffeic acid **3**, rutin **4**, rosmarinic acid **5**, quercetin **6** and palmitic acid **7** (Fig. 1).



156



157

158 *Fig. 1. Structures and ELSD chromatographic signals for glucose (1), gallic acid (2), caffeic acid (3), rutin (4),*
159 *rosmarinic acid (5), quercetin (6) and palmitic acid (7).*

160 According to the literature on triphasic solvent systems,[18] five experimental mixtures composed of *n*-
161 heptan, EtOAc, acetonitrile and water were initially tested (Table 1, entries 1-5). Under those five
162 experimental conditions, the resultant triphasic systems are stable and suitable to dissolve our mixture
163 of control compounds. The distribution of the natural substances in each phase (apolar, intermediate

164 and polar) was then determined by measuring area under the curve (AUC) using HPLC with ELSD
 165 detector (Table 2, entries 1-5). Interestingly, the intermediate phase was found too apolar to efficiently
 166 solubilize most of the targeted molecules whereas this phase is expected to extract the majority of the
 167 usually bioactive secondary metabolites. Notably, most of the rutin stays in the polar phase with a
 168 maximum of 13 % found in the intermediate phase, and, to a lesser extent, gallic acid is only partially
 169 extract in the intermediate phase (32 to 74 %).

170

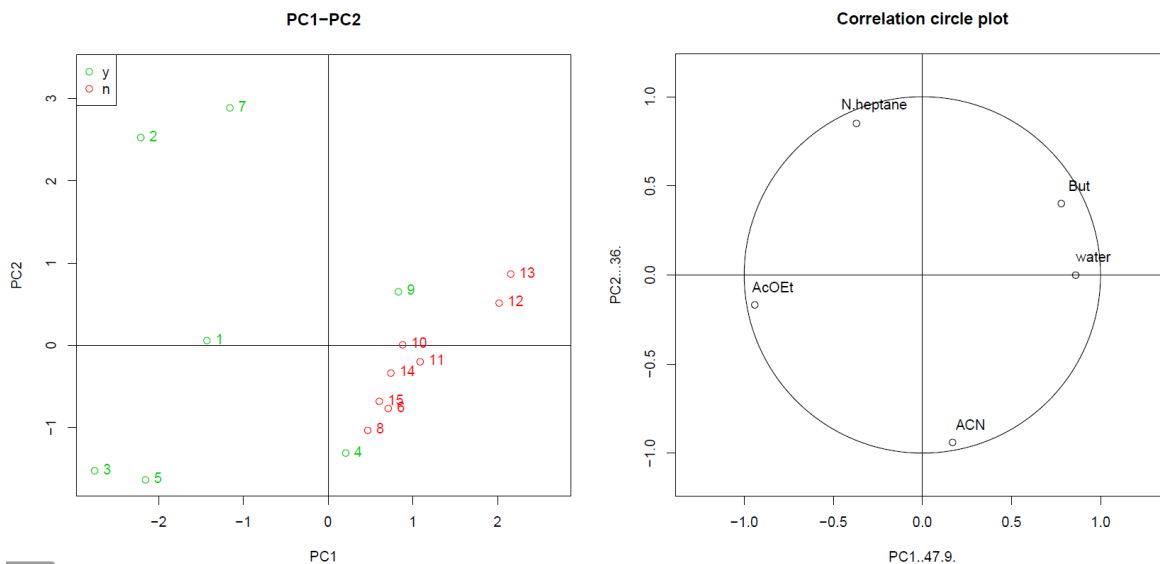
171 *Table 1. Initial composition in n-heptan, EtOAc, acetonitrile, water and butan-1-ol (% in volume) to obtain the*
 172 *triphasic solvent systems used for the development of the methodology.*

| Experiment | n-Heptan (%) | EtOAc (%) | Acetonitrile (%) | Water (%) | Butan-1-ol (%) | Stable triphasic system (Y/N) |
|-------------------|---------------------|------------------|-------------------------|------------------|-----------------------|--------------------------------------|
| 1 | 28.6 | 21.4 | 28.6 | 21.4 | 0 | Y |
| 2 | 40 | 20 | 20 | 20 | 0 | Y |
| 3 | 25 | 25 | 37.5 | 12.5 | 0 | Y |
| 4 | 25 | 12.5 | 37.5 | 25 | 0 | Y |
| 5 | 18.8 | 31.2 | 31.2 | 18.8 | 0 | Y |
| 6 | 23.1 | 11.5 | 34.6 | 23.1 | 7.7 | N |
| 7 | 35.3 | 17.7 | 17.7 | 17.7 | 11.8 | Y |
| 8 | 24 | 12 | 36 | 24 | 4 | N |
| 9 | 26.1 | 13 | 26.1 | 26.1 | 8.7 | Y |
| 10 | 24 | 12 | 30 | 24 | 10 | N |
| 11 | 24 | 10 | 32 | 24 | 10 | N |
| 12 | 25 | 5 | 30 | 25 | 15 | N |
| 13 | 25 | 5 | 28 | 25 | 17 | N |
| 14 | 24 | 12 | 32 | 24 | 8 | N |
| 15 | 24 | 12 | 34 | 24 | 6 | N |
| 16 | 24 | 16 | 30 | 24 | 6 | Y |
| 17 | 20 | 20 | 34 | 24 | 2 | Y |
| 18 | 20 | 20 | 30 | 24 | 6 | Y |
| 19 | 20 | 20 | 27 | 24 | 9 | Y |
| 20 | 20 | 20 | 29 | 24 | 7 | Y |
| 21 | 26 | 13 | 24 | 28 | 9 | Y |
| 22 | 26 | 11 | 28 | 26 | 9 | N |
| 23 | 24 | 13 | 28 | 26 | 9 | Y |
| 24 | 26 | 15 | 26 | 26 | 7 | Y |
| 25 | 24 | 11 | 30 | 26 | 9 | N |
| 26 | 24 | 11 | 29 | 27 | 9 | N |
| 27 | 26 | 17 | 25 | 22 | 9 | Y |
| 28 | 25 | 14 | 27 | 25 | 9 | Y |
| 29 | 19 | 13 | 30 | 26 | 12 | N |
| 30 | 22 | 14 | 29 | 26 | 9 | Y |
| 31 | 22 | 14 | 29 | 27 | 8 | Y |
| 32 | 22 | 14 | 28.5 | 26.5 | 9 | Y |

173

174 In order to enhance the polarity of the intermediate phase, butan-1-ol was added to the initial mixture
 175 to provide new triphasic systems. Since butan-1-ol is more polar than EtOAc and immiscible with
 176 water, it makes the apolar and intermediate phases more polar. Ten new experimental systems
 177 composed of n-heptan, EtOAc, acetonitrile, butan-1-ol and water were then tested (Table 1, entries 6-
 178 15). Unfortunately, most of these experiments failed to give any stable triphasic systems. In order to
 179 further improve our extracting mixture of solvents, a principal component analysis (PCA) was initiated
 180 to visualize all five variables (solvent proportions) in two dimensions and determine a target area
 181 allowing the formation of a suitable triphasic system (Fig. 2). Based on the 15 experiments already
 182 conducted, the PCA allowed us to predict a stable triphasic zone plotted in green in the figure 2.
 183 Based on this analysis, 17 new designed mixtures were prepared (Table 2, entries 16-32) and 13 of
 184 them gave stable triphasic system. The latter were evaluated for their capability to dissolve and

185 separate the mixture of control compounds. In particular, the ability of the intermediate phase to
 186 solubilize substances of varying polarity was monitored (Table 2, entries 7, 9, 16-21, 23a, 24, 27, 28a,
 187 30, 31a and 32a). Among all these tested conditions, the experiments 9, 23, 28a, 30, 31a and 32a
 188 have shown the best extraction results for the intermediate phase suggesting the possibility to
 189 subsequently extract a large diversity of secondary metabolites without extracting substantial amount
 190 of glucose. Indeed, under these conditions, the rutin and gallic acid extractions in the intermediate
 191 phase were substantially increased (35-54 % and 68-80 % respectively). Among these seven
 192 conditions, 30 was not further evaluated because of its similarity in composition with 32.



193
 194 *Fig. 2. Principal component analysis (PCA) of the experiments and the correlation circle of the five variables.*

195
 196 To pursue the reliability of these triphasic systems more thoroughly, the mixture of seven control
 197 compounds was supplemented with saponins. This family of natural compounds is commonly present
 198 in plants and known for its amphiphilic properties able to make emulsions that can easily destabilize
 199 the triphasic system.

200 After addition of aescin to the model extract, two of the five selected conditions (9 and 23) were no
 201 longer forming a sufficiently stable triphasic system (under time or temperature conditions) due to
 202 emulsion and were rejected. For the last three systems (28, 31, 32) with very close composition, the
 203 new distribution of the natural substances in each phase was again determined by HPLC with ELSD
 204 detector (Table 2, entries 28b, 31b and 32b). The condition 28b was not selected due to the high
 205 glucose concentration in the intermediate phase. Taking the experimental uncertainties into account,
 206 the difference between the percentage of experiments 31 and 32 could not be considered as relevant.
 207 Finally, for the next stage of the study, the mixture of solvents of the experiment 31 has been used: *n*-
 208 heptan/EtOAc/Acetonitrile/butan-1-ol/water (22:14:29:27:8). After decantation, the three different
 209 phases were separated: the upper phase (25% of the initial volume) is apolar, the middle phase (48%)
 210 is of intermediate polarity and the lower phase (27%) is the more polar one. The composition of each
 211 phase was determined by ¹H NMR in order to be able to prepare thereafter each phase separately
 212 (Table 3).

213

214 Table 2. Distribution (%) of glucose, gallic acid, caffeic acid, rutin, rosmarinic acid, quercetin, aescin and palmitic acid in the apolar (Ap.), intermediate (Int.) and polar (Pol.)
 215 phases of the various triphasic systems

| Exp. | Glucose | | | Gallic acid | | | Caffeic acid | | | Rutin | | | Rosmarinic acid | | | Quercetin | | | Aescin | | | Palmitic acid | | |
|------|---------|------|------|-------------|------|------|--------------|------|------|-------|------|------|-----------------|------|------|-----------|------|------|--------|------|------|---------------|------|------|
| | Ap. | Int. | Pol. | Ap. | Int. | Pol. | Ap. | Int. | Pol. | Ap. | Int. | Pol. | Ap. | Int. | Pol. | Ap. | Int. | Pol. | Ap. | Int. | Pol. | Ap. | Int. | Pol. |
| 1 | 0 | 0 | 100 | 0 | 42 | 58 | 0 | 52 | 48 | 0 | 2 | 98 | 0 | 67 | 33 | 0 | 90 | 10 | - | - | - | 45 | 55 | 0 |
| 2 | 8 | 0 | 92 | 0 | 32 | 68 | 0 | 62 | 38 | 0 | 0 | 100 | 0 | 80 | 21 | 0 | 97 | 3 | - | - | - | 68 | 32 | 0 |
| 3 | 0 | 0 | 100 | 0 | 74 | 26 | 0 | 92 | 7 | 0 | 6 | 94 | 0 | 96 | 4 | 0 | 100 | 0 | - | - | - | 36 | 64 | 0 |
| 4 | 0 | 3 | 97 | 0 | 52 | 48 | 0 | 71 | 29 | 0 | 13 | 87 | 0 | 80 | 20 | 0 | 92 | 8 | - | - | - | 77 | 23 | 0 |
| 5 | 0 | 0 | 100 | 0 | 38 | 62 | 0 | 75 | 25 | 0 | 0 | 100 | 0 | 88 | 12 | 0 | 100 | 0 | - | - | - | 12 | 88 | 0 |
| 7 | 0 | 5 | 95 | 0 | 63 | 37 | 3 | 82 | 15 | 0 | 21 | 79 | 0 | 89 | 11 | 1 | 97 | 2 | - | - | - | 74 | 25 | 1 |
| 9 | 0 | 24 | 76 | 0 | 67 | 33 | 0 | 76 | 24 | 0 | 48 | 52 | 0 | 77 | 23 | 0 | 91 | 9 | - | - | - | 79 | 18 | 3 |
| 16 | 0 | 0 | 100 | 0 | 62 | 38 | 0 | 84 | 16 | 0 | 28 | 72 | 0 | 84 | 16 | 0 | 97 | 3 | - | - | - | 59 | 40 | 1 |
| 17 | 0 | 0 | 100 | 0 | 44 | 56 | 0 | 77 | 23 | 0 | 4 | 96 | 0 | 81 | 19 | 0 | 98 | 2 | - | - | - | 42 | 57 | 1 |
| 18 | 0 | 2 | 98 | 0 | 54 | 46 | 0 | 79 | 21 | 0 | 9 | 91 | 0 | 85 | 15 | 0 | 98 | 2 | - | - | - | 39 | 61 | 0 |
| 19 | 0 | 2 | 98 | 0 | 65 | 35 | 0 | 85 | 15 | 0 | 16 | 84 | 0 | 89 | 11 | 0 | 99 | 1 | - | - | - | 43 | 57 | 0 |
| 20 | 0 | 2 | 98 | 0 | 62 | 38 | 0 | 79 | 21 | 0 | 16 | 84 | 0 | 84 | 16 | 0 | 99 | 1 | - | - | - | 47 | 52 | 1 |
| 21 | 0 | 7 | 93 | 0 | 64 | 36 | 0 | 78 | 22 | 0 | 20 | 80 | 0 | 82 | 18 | 0 | 96 | 4 | - | - | - | 80 | 19 | 1 |
| 23 | 0 | 35 | 65 | 0 | 76 | 24 | 0 | 82 | 18 | 0 | 48 | 52 | 0 | 82 | 18 | 0 | 94 | 6 | - | - | - | 86 | 12 | 2 |
| 24 | 0 | 1 | 99 | 0 | 62 | 38 | 0 | 77 | 23 | 0 | 15 | 85 | 0 | 86 | 14 | 0 | 98 | 2 | - | - | - | 81 | 17 | 2 |
| 27 | 14 | 6 | 80 | 0 | 76 | 24 | 0 | 90 | 10 | 0 | 35 | 65 | 0 | 95 | 5 | 0 | 99 | 1 | - | - | - | 64 | 34 | 2 |
| 28a | 2 | 21 | 77 | 0 | 80 | 20 | 0 | 81 | 19 | 0 | 44 | 56 | 0 | 89 | 11 | 0 | 97 | 3 | - | - | - | 84 | 16 | 0 |
| 30 | 2 | 31 | 67 | 0 | 75 | 25 | 0 | 84 | 16 | 0 | 50 | 50 | 0 | 91 | 9 | 0 | 97 | 3 | - | - | - | 78 | 22 | 0 |
| 31a | 0 | 12 | 88 | 0 | 68 | 32 | 0 | 81 | 19 | 0 | 35 | 65 | 0 | 88 | 12 | 0 | 96 | 4 | - | - | - | 80 | 20 | 0 |
| 32a | 0 | 17 | 83 | 0 | 72 | 28 | 0 | 83 | 17 | 0 | 43 | 57 | 0 | 87 | 13 | 0 | 96 | 4 | - | - | - | 75 | 23 | 2 |
| 28b | 0 | 46 | 54 | 0 | 71 | 29 | 0 | 81 | 19 | 0 | 54 | 46 | 0 | 79 | 21 | 0 | 87 | 13 | 0 | 68 | 32 | 79 | 19 | 2 |
| 31b | 0 | 26 | 74 | 0 | 67 | 34 | 0 | 72 | 28 | 0 | 43 | 57 | 0 | 73 | 27 | 0 | 88 | 12 | 0 | 53 | 47 | 79 | 20 | 1 |
| 32b | 0 | 29 | 71 | 0 | 66 | 34 | 0 | 76 | 24 | 0 | 48 | 52 | 0 | 75 | 25 | 0 | 91 | 9 | 0 | 58 | 42 | 78 | 19 | 3 |

217

218 Table 3. Composition (% volume) of each phase of the triphasic system 31 in *n*-heptan, EtOAc, acetonitrile, water
 219 and butan-1-ol – ¹H NMR determination.

| | <i>n</i> -Heptan (%) | EtOAc (%) | Acetonitrile (%) | Water (%) | Butan-1-ol (%) |
|---------------------|----------------------|-----------|------------------|-----------|----------------|
| Apolar | 87 | 6 | 5 | 0 | 2 |
| Intermediate | 4 | 21 | 43 | 19 | 13 |
| Polar | 0 | 6 | 28 | 60 | 6 |

220

221 3.2 Evaluation of this new triphasic system and comparison to other extraction methods

222 In order to assess the effectiveness of the developed triphasic system, the 'mixed method' was
 223 compared to SE in MeOH, and to a 'conventional method' involving a successive extraction with five
 224 solvents of increasing polarity, namely cyclohexane, dichloromethane, EtOAc, MeOH and water. This
 225 study was carried out on a model plant, *A. vulneraria*, previously dried and crushed using standard
 226 techniques of phytochemistry (see materials and methods part). Two main parameters were evaluated
 227 in this study: the kinetics of extraction for each phase employed during the process, which is of special
 228 interest to optimize the length of the extraction period, and the total yield of each method. Other critical
 229 parameters were held constant for the extraction, for instance, the particle size of the plant material
 230 and the temperature during the experiments. Concerning the solvent to dry weight sample ratio,
 231 previous studies generally reported an optimal value of 10:1 (v/w).[8] This ratio was used in this study,
 232 except when the wettability properties did not allow it: in this case, the ratio was increased to 15:1 or
 233 20:1 (v/w) if the latter was not sufficient. It should be noted that the SE cannot be used in comparison
 234 with the mixed method in the kinetic study since the experimental volume varies over time due to
 235 siphoning cycles. However, this procedure was compared to the others ('conventional' and 'mixed'
 236 methods) for the yield study.

237

238 3.2.1. Kinetics of extraction processes

239 Tracer compounds identified in the plant material was used as a reference to compare the kinetics of
 240 extraction for the mixed and the conventional methods. The compounds were chosen regarding their
 241 presence as an isolated signal in the HPLC-UV profile: it was then identified by its retention time (RT)
 242 in the table 4.

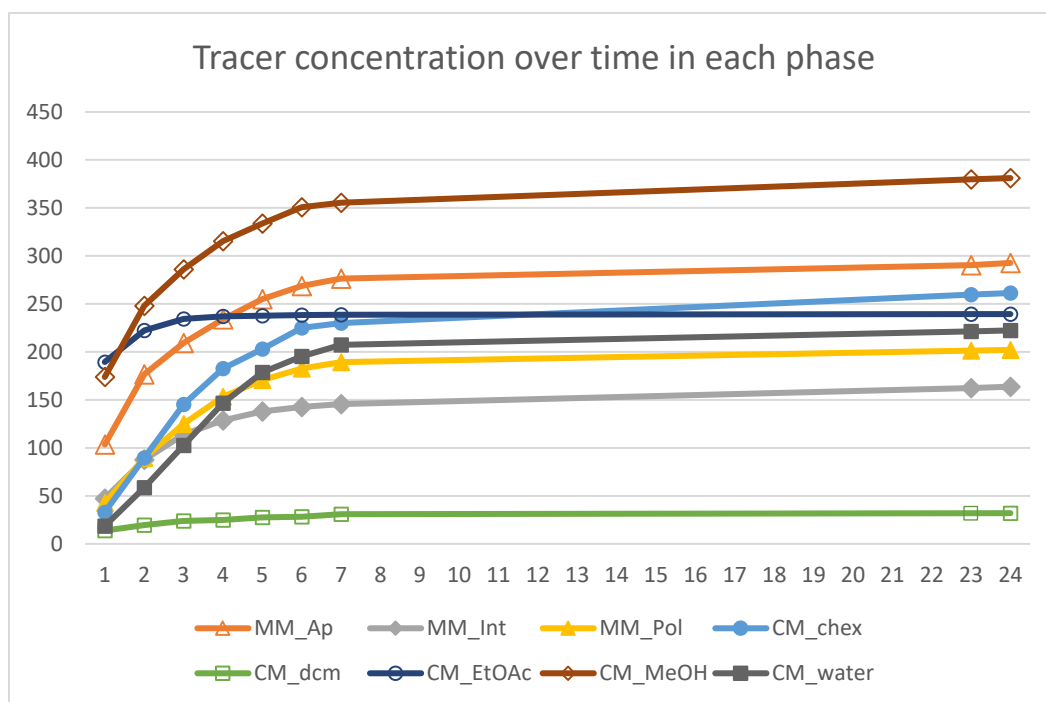
243 Table 4. Retention time (min) of each tracer in the different phase of methods (MM_Ap, MM_Int, MM_Pol: apolar,
 244 intermediate and polar phases of the mixed method respectively; CM_chex, CM_dcm, CM_EtOAc, CM_MeOH,
 245 CM_water: cyclohexane, dichloromethane, EtOAc, MeOH and water phases of the conventional method
 246 respectively)

| RT (min) | MM_Ap | MM_Int | MM_Pol | CM_chex | CM_dcm | CM_EtOAc | CM_MeOH | CM_water |
|----------|-------|--------|--------|---------|--------|----------|---------|----------|
| 9.41 | X | | | X | | | | |
| 6.68 | | | | | X | | | |
| 6.03 | | | | | | X | | |
| 5.79 | | X | | | | | X | |
| 2.13 | | | X | | | | | X |

247

248 The concentration variation of these different tracers over time was thus determined by measuring
 249 AUC in HPLC-UV (Fig. 3). The results obtained in this study indicated that, whatever the solvent and
 250 the method used for the extraction, the saturation effect quickly happens, as early as 1 h for the
 251 dichloromethane and EtOAc extractions of the conventional methods. In any case, after 7 to 8 h of
 252 stirring at 35 °C, the curve flattens indicating that overnight extraction is optimal.

253



254

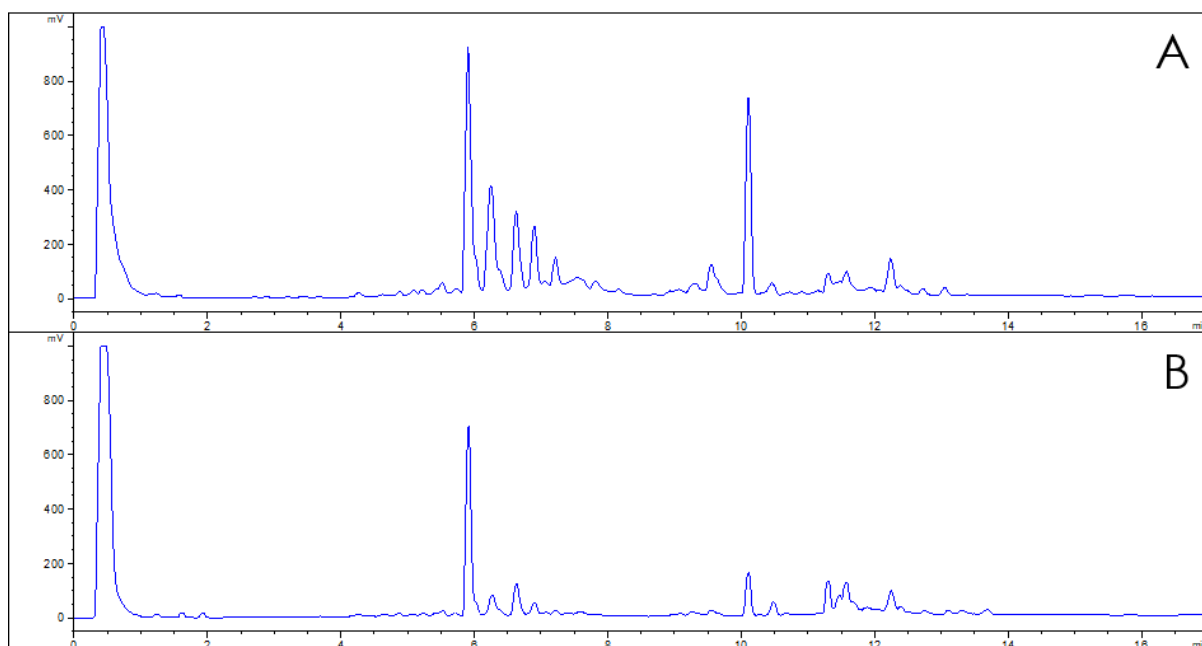
255 *Fig. 3. Concentration (in mAU) of a tracer compound as a function of time (h) for the conventional and the mixed*
 256 *methods (CM and MM respectively)*

257

258 3.2.2. Yields of the various extractions

259 Here, three methods were compared with respect to their yield to assess the effectiveness of the
 260 triphasic system: the mixed method, the conventional method using five solvents and the SE. The
 261 yield of extraction was here determined after 24 h of stirring at 35 °C (except for the SE carried out in
 262 refluxing MeOH) of the plant material with the different solvents or mixture of solvents (Table 3).

263 As part of the establishment of a non-targeted methodology able to extract a large variety of
 264 metabolites, a first global comparison between the yields achieved for the various experiments shows
 265 a significant difference for SE (total yield of 15 %) and the two other methodologies (total yield of more
 266 or less 30 %) even though the number of successive extraction is obviously different in these three
 267 protocols. When now considering the valuable secondary metabolites, they are generally found in the
 268 intermediate phases while primary metabolites fatty acids and carbohydrates constitute the main part
 269 of molecules found in the apolar and polar phases respectively. It is thus more relevant to compare the
 270 yield obtained for the intermediate phase of the mixed method (about 14%) to the cumulative
 271 extraction yield of the EtOAc and MeOH phase of the conventional method (about 9 %) and to those
 272 of the SE (about 15%). This study clearly shows here a more effective extraction from the mixed
 273 method and SE compare to the conventional method. To compare more deeply the last two extraction
 274 procedures, the chromatographic profiles of the intermediate phase of the mixed method and SE were
 275 investigated (Fig. 4). The higher diversity content in secondary metabolites displayed in the profile of
 276 the intermediate phase of the mixed method (A) highlighted the value of our methodology.



277
278 Fig. 4. ELSD chromatographic profile for (A) the intermediate phase of the mixed method and (B) SE

279

280 Table 5. Yields of the three different methods of extraction

| Mixed method (yield %) | | Conventional method (yield %) | | SE (yield %) | |
|------------------------|-------------|---------------------------------|-------------|--------------|-------------|
| Apolar | 2.9 | Cyclohexane | 1.0 | MeOH | 15.3 |
| | | CH ₂ Cl ₂ | 1.2 | | |
| Intermediate | 14.4 | EtOAc | 0.6 | | |
| | | MeOH | 8.3 | | |
| Polar | 15.4 | H ₂ O | 17.5 | | |
| Total | 32.7 | Total | 28.6 | Total | 15.3 |

281

282 4. Conclusion

283 Finally, this study has led to the development of a new maceration technique to extract natural
 284 products using successive extractions by a triphasic system elaborated from a mixture of five solvents:
 285 *n*-heptan/EtOAc/acetonitrile/butan-1-ol/water (22:14:29:27:8). The particular strength of this mixed
 286 method is the good partition of compounds obtained between different phases of extraction. Notably,
 287 the major part of the secondary metabolites are recovered in the intermediate phase while the very
 288 polar (e. g. carbohydrates) and the very apolar (e. g. fatty substances) compounds are well
 289 discriminated and remained in the polar and apolar phases respectively. This relevant result is also
 290 correlated to the good yield obtained during the extraction process. Another critical interest is related
 291 to the time of extraction: it was highlighted here that, whatever the solvent used in the conventional or
 292 the mixed method, a maximum of 8 h allowed the saturation of the solvent. Thus, regarding the
 293 different results obtained for the mixed and the conventional methods, there are clear indications that
 294 the mixed method improves the effectiveness of the process: higher quantity of compounds is
 295 extracted within a shorter time (3 extractions instead of 5 in the conventional method) while using a
 296 lower quantity of solvent. Besides, the characterization of each phase of the triphasic system could
 297 henceforth allow to directly prepare them without having to make the initial mixture – thereby reducing
 298 the total volume of solvent needed, and thus cost. The phase composition of the best triphasic system
 299 was determined by ¹H NMR experiments: *n*-heptan/EtOAc/acetonitrile/butan-1-ol (87:6:5:2) (%
 300 volume) for the apolar phase, *n*-heptan/EtOAc/acetonitrile/water/butan-1-ol (4:21:43:19:13) for the
 301 intermediate phase and EtOAc/acetonitrile/water/butan-1-ol (6:28:60:6) for the polar phase. This study
 302 was first implemented in a model plant, *A. vulneraria*, and then this new methodology was

303 subsequently used for the extraction of 22 different alpine plants harvested in the French Alps, around
304 the Col du Lautaret region and identified thanks to the expertise of the SAJF (Station Alpine Joseph
305 Fourier) botanists. This way, 66 extracts with an average total yield of 32% were obtained, thereby
306 validating this extraction process and proving the effectiveness of this protocole. To conclude, the
307 application of this methodology as a research routine process for extraction could be of particular
308 interest for phytochemists to perform efficient extraction of natural products, especially secondary
309 metabolites. However, this new protocole cannot replace extraction methods used for specific
310 molecular families.

311

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