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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.

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

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

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

2 Materials and methods

2.1 Solvents and chemicals

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

2.2 HPLC and NMR analyses

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

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

2.3 Plant collection and sample preparation

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

2.4 Extraction

2.4.1. Development of the triphasic system

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

2.4.2. Conventional method and/or mixed method

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

2.4.3. Soxhlet extraction procedure

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

3. Results and discussion

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

3.1 Development of a new triphasic solvent system

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

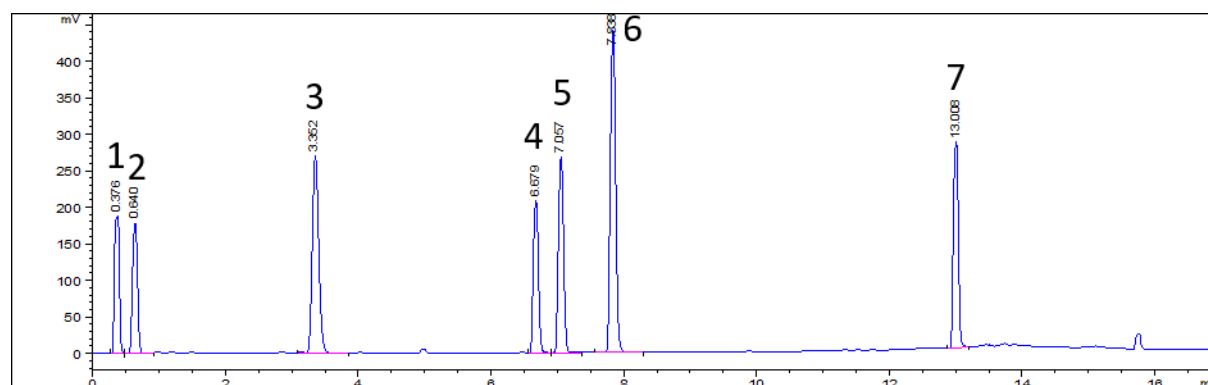
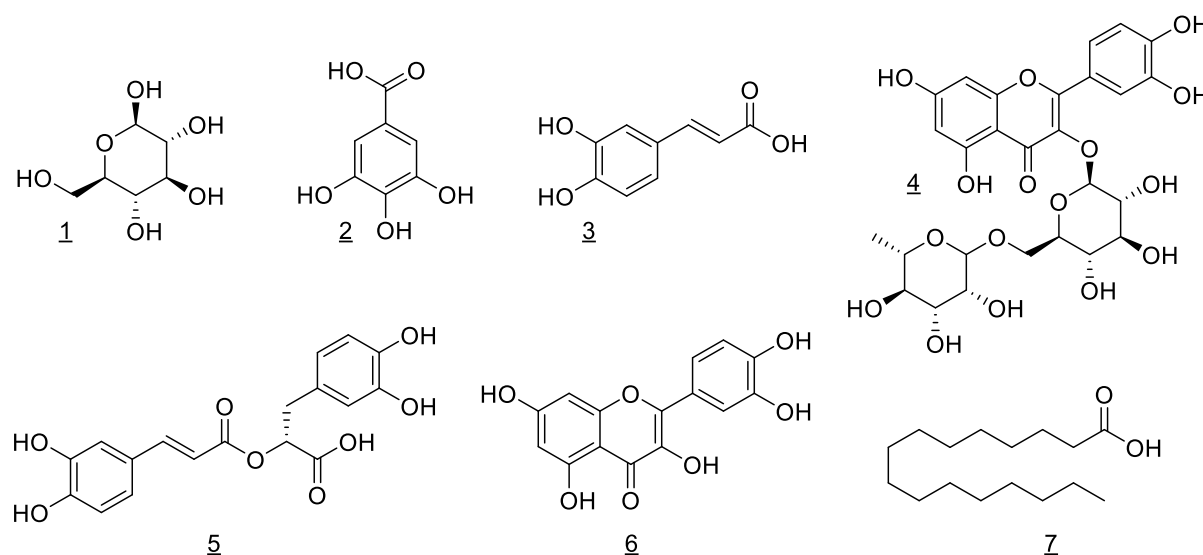


Fig. 1. Structures and ELSD chromatographic signals for glucose (**1**), gallic acid (**2**), caffeic acid (**3**), rutin (**4**), rosmarinic acid (**5**), quercetin (**6**) and palmitic acid (**7**).

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

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

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

Experiment	<i>n</i> -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

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

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

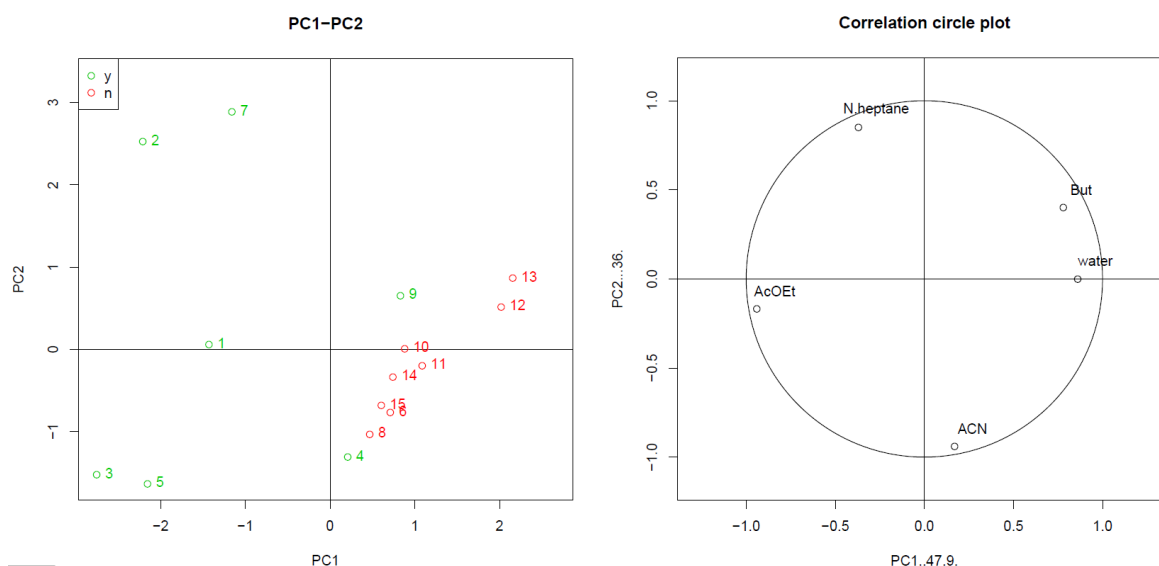


Fig. 2. Principal component analysis (PCA) of the experiments and the correlation circle of the five variables.

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

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

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

Table 3. Composition (% volume) of each phase of the triphasic system 31 in *n*-heptan, EtOAc, acetonitrile, water 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

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

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

3.2.1. Kinetics of extraction processes

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

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

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

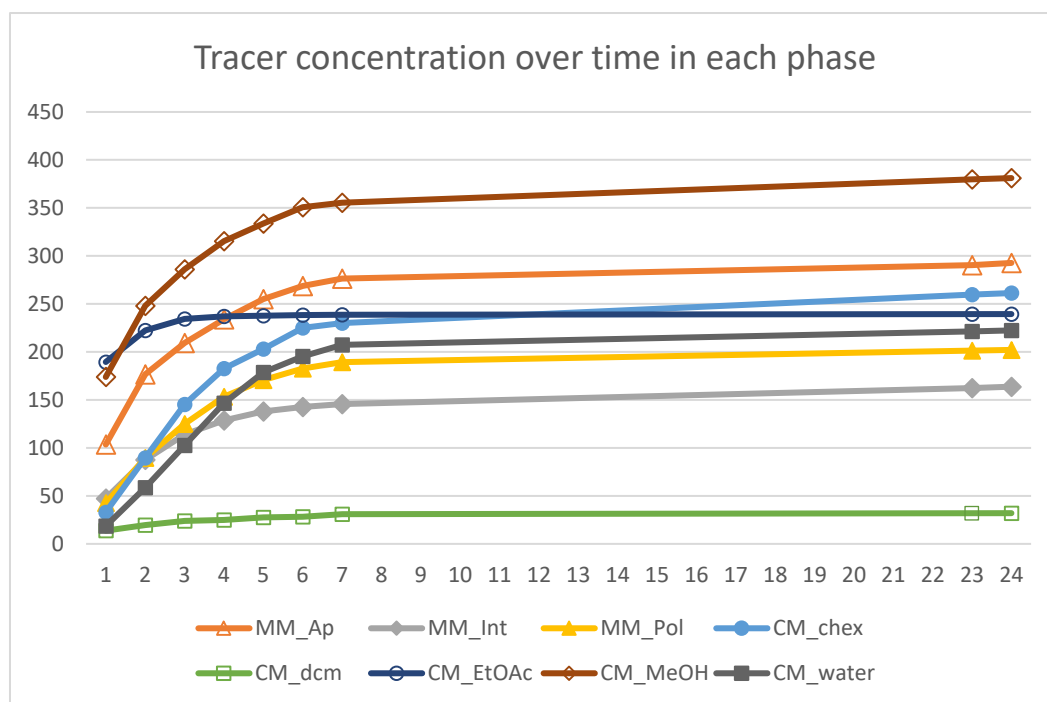


Fig. 3. Concentration (in mAU) of a tracer compound as a function of time (h) for the conventional and the mixed methods (CM and MM respectively)

3.2.2. Yields of the various extractions

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

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

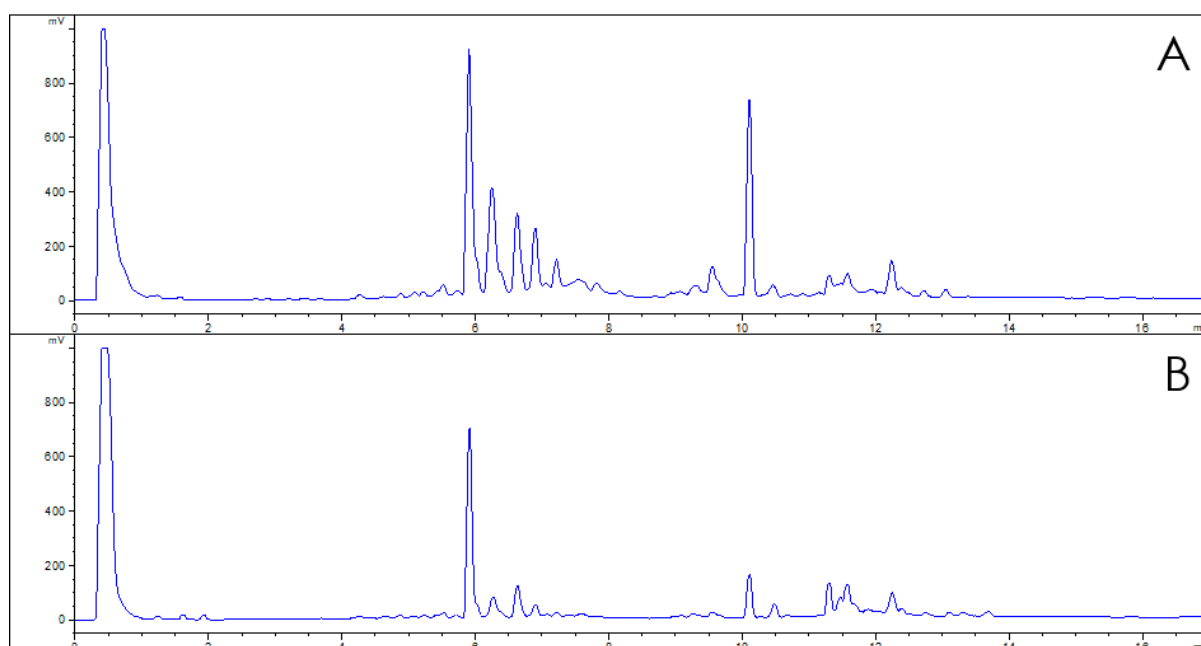


Fig. 4. ELSD chromatographic profile for (A) the intermediate phase of the mixed method and (B) SE

Table 5. Yields of the three different methods of extraction

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

4. Conclusion

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

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

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