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Advantages of Soxflo Extractions for Phytochemical Analysis and Bioassay Screening. 1. Terpenoids

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The Soxflo technique was evaluated for the rapid extraction of plant materials (<90 min) at room temperature. Qualitatively similar chromatograms were obtained by gas chromatography and thin layer chromatography (TLC) with Soxflo (SoF) and Soxhlet (SoL) extracts. Sequential solvent extractions by SoF gave slightly higher yields (132%) of five major sesquiterpenoids. TLC revealed that SoF extractions at room temperature were more selective as extracts contained compounds with a narrower range of R_f values. This means that the SoF technique offers the potential for one-step extractions and partial fractionation. This study also showed that there were large differences in the volatile composition of dried and fresh *Piper* fruits: dried fruits had predominantly sesquiterpenoids while fresh fruits had considerable quantities of both mono- and sesquiterpenoids. This is the first report of α-guaiene and α- and β-selinene in *Piper guineense* fruits. It is suggested that the SoF technique can be useful for the screening of large numbers of plants for phytochemicals or for the preparation of plant extracts for subsequent bioassay studies.

KEYWORDS: Dry column procedure; plant screening; bioassays; environment friendly extractions; volatile oils; *Piper guineense*; *Khaya grandifoliola*

INTRODUCTION

Natural products can be extracted from plants by several techniques. Recent reviews (*1*, *2*) compared in detail methods based on solvent extraction, supercritical fluid extractions, microwave-assisted extraction, headspace analysis, purge and trap techniques, solid-phase microextraction, direct thermal desorption, and steam distillation−solvent extractions.

To summarize briefly, solvent extractions at elevated temperature using the standard Soxhlet apparatus tend to yield more than other methods but are prone to contamination from coextracted lipids, artifact formation, or losses of labile or volatile compounds (*3*−*10*). Chaintreau (*2*), therefore, stressed the importance of extractions at room temperature (rt) to avoid artifacts.

Solvent extractions at rt can give good recoveries of compounds with a wide range of polarities and volatilities (*10*). Plant materials have been extracted at rt by shaking, sonicating, or simply leaving them standing in a solvent for several hours. Oils obtained by these solvent extractions tend to reflect a plant's natural odor very well (*11*). Solvent extractions at rt are commonly employed for extracting biologically active compounds or volatile oils in chemotaxonomic plant surveys and in bioassay studies (*11*−*14*). Supercritical fluid extraction with CO_2 is also capable of preserving unstable or heat-sensitive compounds (*11*). It is a powerful, although expensive, technique for extracting highly volatile compounds (*15*); yields tend to be higher than those from steam distillation but lower than those from solvent extraction techniques (*7*, *9*).

In contrast, steam distillation is widely used in industry for the recovery of essential oils (*11*, *16*). For the aforementioned reasons, this technique is not recommended for phytochemical research or bioassay studies of botanicals or therapeutic compounds (*6*, *15*, *17*). The above studies have shown that the qualitative and quantitative composition of extracts tends to differ depending on the extraction technique and concluded (*1*, *7*) that there are few methods currently available for extracting flavors and volatiles that are simultaneously cheap, easy, and good.

We recently developed the Soxflo technique, a dry column procedure, for extracting fat from foods and animal feeds (*18*). It proved to be an efficient, yet rapid (ca. 1 h), technique in which a solvent is simply passed once through a sample that is packed into the form of a column. As extractions take place at room temperature, it is a mild and environmentally friendly technique requiring neither heating nor cooling water. The simplicity of the design makes it a user-friendly technique that produced excellent yields with 50% lower relative standard deviations than Soxhlet extractions. The present study examines the suitability of the Soxflo (SoF) technique for extracting terpenoids and compares it to the classical Soxhlet (SoL) technique by sequential solvent extractions (*1*, *18*).

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Table 1. Mean Weights (mg) Obtained from *P. guineense* Fruits (5 g) and *K. grandifoliola* Bark (5 g) by Sequential SoF or SoL Extractions[a]

	hexane		ethyl acetate		acetone		ethanol		methanol		overall results	
	SoF	SoL	SoF	SoL	SoF	SoL	SoF	SoL	SoF	SoL	SoF	SoL
						P. guineense						
mean	153.8	267.6	216.1	137.8	45.3	43.6	37.1	63.2	80.8	100.5	sum total = 533.2	sum total = 612.6
SD	7.22	21.92	8.56	16.05	3.62	7.96	1.9	2.62	2.2	7.65		
RSD	4.69	8.19	3.96	11.65	7.99	18.26	5.12	4.15	2.72	7.61	av RSD = 4.90	av RSD = 9.97
t-value	11.02		9.63		0.43		18.00		5.51			
sig level, *P*<	0.001		0.001		n.s.		0.001		0.001			
						K. grandifoliola						
mean	19.2	41.0	41.4	115.8	165.5	363.5	202.7	368.2	190.9	204.7	sum total = 619.7	sum total = 1093.2
SD	0.81	1.14	0.85	5.05	4.62	10.12	8.11	81.32	2.19	9.93		
RSD	4.21	2.78	2.05	4.36	2.79	2.78	4.00	22.09	1.15	4.85	av RSD = 2.84	av RSD = 7.37
t-value	27.06		25.16		30.82		3.51		2.35			
sig level, *P*<	0.001		0.001		0.001		0.03		n.s.			

[a] SD = standard deviation, RSD = relative standard deviation.

MATERIALS AND METHODS

Reagents. The following reagents or authentic standards were purchased: acetone (distol grade), ethanol (Analar grade), ethyl acetate (analysis grade), hexane (residue analysis grade), methanol (HPLC grade) (Fisher Scientific, Loughborough, U.K.); β-caryophyllene (Fluorochem, Old Glossop, U.K.), caryophyllene oxide (Sigma-Aldrich, Poole, U.K.), guaiene (Greyhound Chromatography & Allied Chemicals, Birkenhead, U.K.).

Plant Materials. Seeds of *Piper guineense* (Piperaceae) were obtained from a local market in Bumenda, North-West Province, Cameroon. Samples were finely ground (<1 mm) in a Janke and Kunkel A10 grinder (IKA Labortechnik, Staufen, Germany). Bark of *Khaya grandifoliola* (Meliaceae) was from Makurdi, Benue, Nigeria. Samples were finely ground (<1 mm) and stored in the dark.

Soxflo Extractions. Powdered samples (5.0 g) were placed between two cellulose disks in the stainless steel sample holder (25 mm diameter, 65 mm length) of the Soxflo instrument (Scientific & Technical Supplies Ltd., Newmarket, U.K.; for a schematic view of the SoF extractor unit see ref 18) and firmly compressed by hand as described previously (*18*). The sample holder is then inserted into the SoF apparatus which consists of (i) a solvent reservoir at the top that is connected to a small pump, (ii) a tightly fitting support for the sample holder, and (iii) a connector for a round-bottom flask to collect the eluant. Replicate samples (five for *Piper* and three for *Khaya* samples) were extracted successively with 70 mL of each of hexane, ethyl acetate, acetone, ethanol, and methanol at a flow rate of 1 drop/s [extractions lasted between 60 and 90 min]. Note: each solvent (70 mL) is passed just once through the column of plant material. Any residual solvent is forced out by a peristaltic pump before applying the next solvent. Solvents were removed on a rotary evaporator below 35 °C, and the residues were weighed.

Soxhlet Extractions. Samples (5.0 g) were placed into thimbles (28 mm diameter, 80 mm length), and replicates (five for *Piper* and three for *Khaya* samples) were extracted successively with 150 mL of each solvent at the following cycling rates: hexane (1 cycle per 9 min), ethyl acetate (1 per 14), acetone (1 per 10), ethanol (1 per 13), and methanol (1 per 14). Each solvent extraction lasted for 7 h. Solvents were removed as described above. Thimbles containing the samples were air-dried at rt before adding the next solvent.

Gas Chromatography−Mass Spectrometry (GC−MS). Dried extracts were dissolved in dichloromethane (5 mg/mL), and 3 μL samples were injected into the GC−MS system (Carlo Erba GC8000 interfaced with a Fisons MD800 quadrupole mass spectrometer). The GC instrument was fitted with a split−splitless injector (the split ratio was 25:1) and equipped with a Restek RTX-5 column (60 m × 0.25 mm i.d., 0.25 μm film thickness, catalog no. 10226, Bellefonte, PA). The injector temperature was 250 °C; the oven temperature was 80 °C for the first 5 min and was then programmed to rise at 6 °C/min from 80 to 270 °C. Helium was used as the carrier gas set at 140 kPa; the flow rate was 1.2 mL/min. The interface temperature was 251 °C. MS conditions were as follows: EI +ve mode; ionization energy, 70 eV; ion source temperature, 200 °C; source current, 967 μA; trap current, 122 μA; filament current, 4.26 A; scan rate, 2 scans per s with 50−210 amu/scan.

Characterization of compounds was based on GC retention times, computer matching with the Wiley6 library (acceptable matches, >900), visual comparison of the fragmentation patterns, and comparison with authentic compounds.

Thin Layer Chromatography (TLC). Crude SoF and SoL extracts were redissolved in the solvents used for respective extractions (at 2 mg/mL); 5 μL samples were applied to TLC plates (Si60, 10 × 20 cm, Merck number 105553) (Merck, Darmstadt, Germany), developed with hexane−ethyl acetate-methanol (HEM, 60:40:1, v/v) or chloroform-methanol−water (CMW, 73:24:3), and detected with either an anisaldehyde−glacial acetic acid−sulfuric acid spray (0.5:50:1) or a vanillin−sulfuric acid spray (8 mL of ethanol is added to a cooled mixture of 0.5 g of vanillin in 2 mL of concentrated sulfuric acid) (*17*). Colors were revealed with a heat gun.

Statistical Analysis. Data were subjected to *t*-tests using INSTAT (*19*).

RESULTS AND DISCUSSION

To evaluate the efficiency of SoF extractions, we chose two contrasting plant materials. The genus *Piper* contains considerable quantities of volatiles (mono- and sesquiterpenoids) (*20*−*22*), and the genus *Khaya* is known for its nonvolatile triterpenoids (limonoids) (*23*−*24*).

Sequential Solvent Extractions by SoF and SoL. SoF extractions at room temperature yielded a total of 533 mg (87%) from *P. guineense*, compared to 613 mg for SoL extractions at elevated temperatures, but only 620 mg (57%) compared to 1093 mg for SoL from *K. grandifoliola* (**Table 1**). The reproducibility of the SoF procedure was better: average relative standard deviation (RSD) values of SoF were less than half of those of SoL extractions as reported previously for fat extractions (*18*). Relatively high variations for SoL extractions are not uncommon (*2*).

The total quantities extracted by the two methods differed significantly for all solvents, except for the *Piper* acetone and *Khaya* methanol extracts. Closer examination of the *Piper* extracts revealed that the sums of hexane plus ethyl acetate extracts were comparable for SoF (0.37 g) and SoL (0.41 g). Furthermore, although statistically significant, the SoL ethanol and methanol fractions extracted only 20 mg more than SoF from 5 g of plant material.

GC−MS Analysis of *P. guineense* Extracts. GC chromatograms showed that the qualitative compositions of the volatile oils were the same whether extracted by SoF or SoL (**Figure 1**). **Table 2** lists retention times of the GC peaks together with

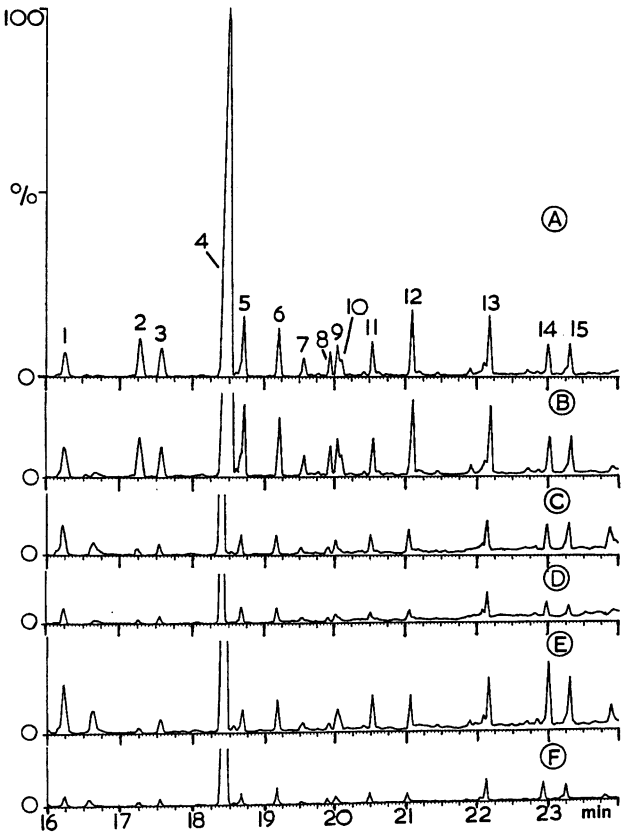

Figure 1. Gas chromatograms of volatile oils from *P. guineense* fruits. Extracts were prepared by sequential extraction with hexane (a, b), ethyl acetate (c, d), and acetone (e, f) using Soxflo (a, c, e) or Soxhlet (b, d, f) extractors. Peak numbers refer to compounds listed in **Table 2**.

their characteristic mass ions and Wiley library matches. Most of these compounds from dried fruits had previously been identified in fresh fruits of *P. guineense* (*20*). In addition, the present study found α- and *β*-selinene plus α-guaiene. Both selinene isomers and *δ*-guaiene have been reported in *P. guineense* leaves (*25*) and α-guaiene has been reported in *P. goessii* leaves (*22*). Varietal or environmental effects may account for these differences.

Table 3 gives the areas of the seven major peaks that were extracted by hexane, ethyl acetate, and acetone. Hexane extracted similar quantities in the SoF and SoL procedure, with one exception: SoL extracted significantly more *δ*-elemene ($P < 0.05$). However, SoF extracted significantly more of all peaks with ethyl acetate and especially acetone. As a result, sequential SoF extractions yielded on average 132% more volatile oils than SoL. The low recoveries in the two SoL extracts were not investigated further and could be a result of degradation during boiling in the polar EtOAc or acetone solvents. Indeed, degradation, oxidation, structural rearrangements, and polymerizations of terpenoids can occur quite readily even under relatively mild conditions, and rt extractions have, therefore, been recommended (*2*, *15*, *17*). It is unlikely that these losses occurred overnight while samples were drying before the next SoL extraction, as the powdered *Piper* samples had been stored for several weeks previously.

Table 1 shows that the combined weights extracted from *Piper* by hexane, ethyl acetate, and acetone in SoF were only 92% of those from the SoL technique; therefore, SoF produced cleaner extracts of volatile oils. The corollary of this is that SoL extracted more of the less volatile compounds which did not appear in gas chromatograms. This is not surprising because Soxhlet extracts of plant samples often contain coextracted lipids (*2*, *10*).

Comparison of Fresh versus Dried *P. guineense* Fruits. Our more recent studies on fresh *P. guineense* fruits found considerable quantities of both mono- and sesquiterpenoids (**Figure 2a**). The monoterpenoids were α-pinene, sabinene, *β*-pinene, α-phellandrene, *δ*-3-carene, limonene, *β*-phellandrene, (*Z*)-*β*-ocimene, α-terpinolene, linalool, camphor, and isoborneol (peaks 1−12, **Figure 2a**). The dried Cameroonian *Piper* fruits had mainly sesquiterpenoids (peaks 13 and above) and hardly any monoterpenoids (peaks 2−6) (**Figure 2b**). *trans*-Caryophyllene accounted for approximately half of the volatile oils in dried fruits (**Table 2**) but for less than 2% in fresh fruits (*20*). These SoF extractions, therefore, indicate that compositional differences between fresh and dried fruits were due to drying rather than the extraction technique.

TLC of *P. guineense* Extracts. In general, SoF and SoL extracts gave similar TLC chromatograms (**Figure 3**). Closer examination revealed, however, that the SoF hexane fraction had more high R_f material than the SoL extract ($R_f > 0.68$, lines 1 and 2) and the SoF ethyl acetate extract had much more material with R_f values of 0.06−0.49 (lines 3 and 4). This indicated that the SoF extracts were more concentrated as equal quantities of the extracts had been applied to the TLC plates.

TLC of *K. grandifoliola* Extracts. SoF extractions produced slightly "cleaner" extracts than SoL (**Figure 4**) as sequential SoF extractions resulted in some fractionation between high and low R_f material. Closer examination of **Figure 4a** shows that

Table 2. Composition of Volatile Oils in Dried *P. guineense* Fruits (see **Figure 1** for GC Traces)

GC peak no.	R_T [min]	% composition[a]	molecular ion (M+)	MS peaks[b]	Wiley match	compound
1	16.238	3.4	204	121; 93, 136, 91	974	*δ*-elemene
2	17.263	3.9	204	119; 105, 161, 93	994	α-copaene
3	17.563	2.8	204	93; 161, 67, 107	991	*β*-elemene
4	18.530	55.7	204	133; 93, 91, 69	921	*trans*-caryophyllene
5	18.714	4.8	204	69; 93, 133, 120	973	*trans*-*β*-farnesene
6	19.206	3.8	204	93; 121, 147, 80	919	α-humulene
7	19.564	1.3	204	105; 93, 106, 91	871	α-guaiene
8	19.939	1.7	204	105; 107, 93, 67	978	*β*-selinene
9	20.039	2.4	204	69; 93, 105, 161	950	*β*-bisabolene
10	20.098	1.1	204	189; 107, 105, 93	980	α-selinene
11	20.540	2.7				ui
12	21.106	5.6	208	208; 193, 133, 177	959	*cis*-isoelemicin
13	22.190	5.6	220	93; 79, 91, 95	956	caryophyllene oxide
14	23.024	2.4				ui
15	23.324	2.7	222	95; 121, 161, 105	936	T-muurolol

[a] Obtained by summation of peak areas. [b] Base peak followed by three other major ions; ui = unidentified.

Table 3. GC–MS Peak Areas Obtained from *P. guineense* by Sequential SoF or SoL Extractions [Total Ion Count]

		hexane		ethyl acetate		acetone		sum total		
GC–MS peak		SoF	SoL	SoF	SoL	SoF	SoL	SoF	SoL	SoF as % of SoL
δ-elemene	mean	39.2	45.7	10.4	1.9	46.7	5.0	96.3	52.6	183
	SD	3.81	3.73	3.45	0.96	17.09	1.04			
	RSD	9.7	8.2	33.2	51.9	36.6	20.9			
	t (sig level)	2.75 ($P < 0.05$)		5.32 ($P < 0.001$)		5.45 ($P < 0.001$)				
α-copaene	mean	49.0	50.4							
	SD	5.03	4.57							
	RSD	10.3	9.1							
	t (sig level)	0.46 (n.s.)								
β-elemene	mean	35.9	37.4	3.0	0.7	10.3	3.5	49.2	41.6	118
	SD	3.90	3.40	0.83	0.29	3.40	1.76			
	RSD	10.8	9.1	27.6	42.1	33.2	50.1			
	t (sig level)	0.66 (n.s.)		5.88 ($P < 0.001$)		3.93 ($P < 0.01$)				
trans-caryophyllene	mean	717.2	737.3	94.4	26.0	326. 5	206.7	1138.1	970.0	117
	SD	48.06	90.81	23.82	9.63	83.44	30.08			
	RSD	6.7	12.3	25.2	37.0	25.6	14.6			
	t (sig level)	0.44 (n.s.)		5.95 ($P < 0.001$)		3.02 ($P < 0.02$)				
trans-β-farnesene	mean	54.8	61.2	3.9	1.0					
	SD	11.90	8.55	1.35	0.39					
	RSD	21.7	14.0	35.0	40.4					
	t (sig level)	0.98 (n.s.)		4.61 ($P < 0.01$)						
humulene	mean	46.8	50.5	4.9	1.1	17.9	6.6	69.6	58.2	120
	SD	4.90	4.47	1.59	0.47	5.44	3.14			
	RSD	10.5	8.9	32.8	41.9	30.3	47.7			
	t (sig level)	1.24 (n.s.)		5.05 ($P < 0.001$)		4.04 ($P < 0.01$)				
cis-isoelemicin	mean	62.2	66.2	4.8	0.9	19.9	5.3	86.9	72.4	120
	SD	6.82	8.03	1.73	0.56	6.79	4.06			
	RSD	11.0	12.1	35.8	59.2	34.1	76.4			
	t (sig level)	0.86 (n.s.)		4.79 ($P < 0.01$)		4.13 ($P < 0.01$)				

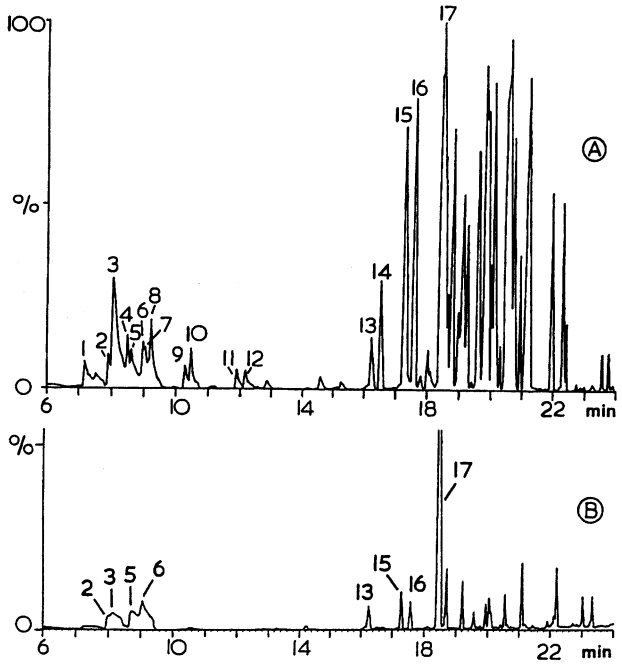

Figure 2. Gas chromatograms of the Soxflo hexane extracts of (a) fresh and (b) dried *P. guineense* fruits. Peak numbers: 1, α-pinene; 2, sabinene; 3, β-pinene; 4, α-phellandrene; 5, δ-3-carene; 6, limonene; 7, β-phellandrene; 8, (*Z*)-β-ocimene; 9, α-terpinolene; 10, linalool; 11, camphor; 12, isoborneol; 13, δ-elemene; 14, α-cubebene; 15, α-copaene; 16, β-elemene; 17, *trans*-caryophyllene.

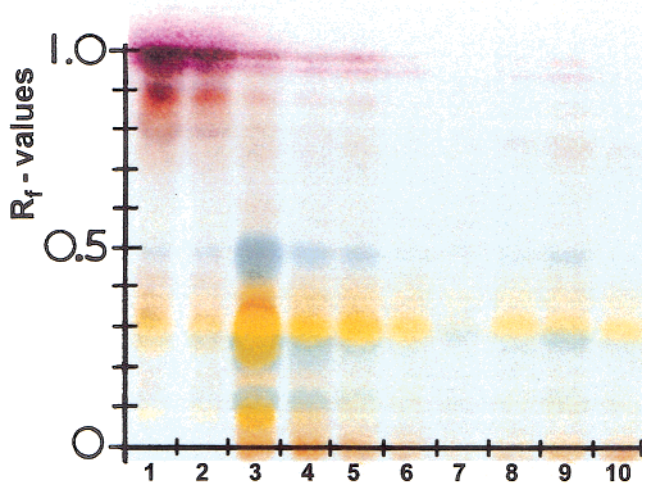

Figure 3. TLC of *P. guineense* extracts developed in HEM and detected with anisaldehyde. Extract numbers are as follows: Soxflo (odd numbers) and Soxhlet (even numbers); hexane (lines 1 and 2), ethyl acetate (lines 3 and 4), acetone (lines 5 and 6), ethanol (lines 7 and 8), and methanol (lines 9 and 10).

the SoF hexane extract (line 11) had much less of a compound near the origin ($R_f = 0.06$) than the corresponding SoL extract (line 12).

SoF and SoL ethyl acetate extracts (lines 13 and 14, **Figure 4a,b**) had comparable amounts for compounds with higher R_f values (i.e., between 0.67 and 0.90). However, the SoF extract (line 13) had less material with R_f of 0.03 (**Figure 4a**, TLC plate eluted with HEM). The SoF extract (line 13) had also noticeably less compounds with lower R_f values (i.e., 0.00 to 0.44; **Figure 4b**, TLC plate eluted with CMW).

The SoF acetone extracts (lines 15, **Figure 4**) had more of the low R_f compounds (0.00−0.44) than SoL extracts (lines 16). This is particularly obvious for compounds with R_f values of <0.11 (**Figure 4b**) and R_f of 0.03 (**Figure 4a**).

Finally, the SoF ethanol extract (line 17, **Figure 4b**) had mostly material with R_f of 0.00−0.08, whereas SoL (line 18) extracted material up to R_f of 0.43 together with a brown streak (R_f of 0.00−0.14). The methanol extracts (lines 19 and 20) produced complex TLC patterns and are not considered further.

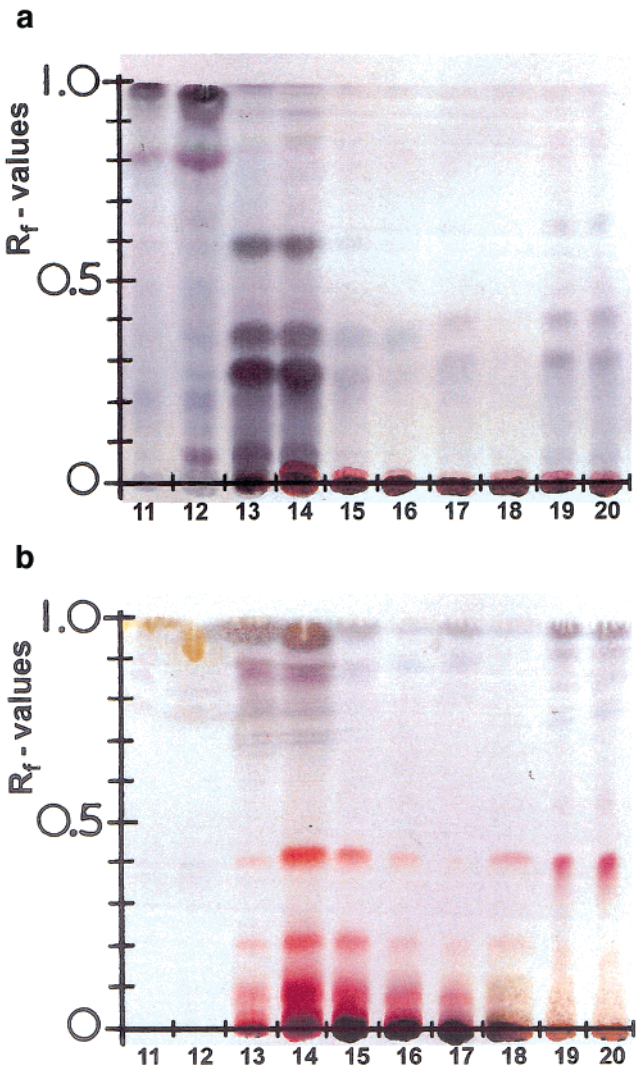

Figure 4. TLC of *K. grandifoliola* extracts developed in two solvents, (a) HEM and (b) CMW, and detected with vanillin. Extract numbers are as follows: Soxflo (odd numbers) and Soxhlet (even numbers); hexane (lines 11 and 12), ethyl acetate (lines 13 and 14), acetone (lines 15 and 16), ethanol (lines 17 and 18), and methanol (lines 19 and 20).

To summarize, SoF extracted relatively more with ethyl acetate of the higher R_f material (>0.67, line 13, **Figure 4b**), subsequent acetone extraction pulled out more material with R_f values of <0.44 (line 15), and ethanol extracted more of the lower R_f material (<0.08, line 17).

These results suggest that sequential SoF extractions at room temperature are slightly more selective by extracting compounds with a narrower range of R_f values. SoF, therefore, offers the possibility for rapid extractions and some preliminary fractionation of natural plant products. This is probably so because SoL extractions operate at elevated temperatures and over many hours, which tends to enhance the solubility of compounds thereby masking solvent selectivities.

Harborne (*17*) pointed out that high boiling impurities are sometimes present in conventional solvent extracts of mono- and sesquiterpenes, and Kerrola (*6*) mentioned that waxes are common in spices. We noticed that some SoF extracts appeared to be visibly cleaner; that is, the extracted colors were brighter and on one occasion crystals formed overnight from the *Piper* SoF but not from the SoL ethyl acetate extract. In the case of *Khaya*, a highly aliphatic substance (^{1}H NMR spectrum not shown) separated out during concentration of the SoL, but not SoF, hexane extract.

To conclude, conventional plant extractions at room temperature are often time consuming, as samples are soaked overnight or extracted several times in order to maximize yields. We indicated previously (*18*) that SoL extractions based on the Nernst partition laws are time consuming and require many extraction cycles to achieve completion. In contrast, the one-directional flow of solvent in the SoF technique exposes the sample matrix constantly to fresh solvent and can be compared to flash column chromatography. This study indicated that the Soxflo procedure can be a useful technique for room-temperature extractions of plants, as it produced yields for terpenoids that were comparable to Soxhlet extractions.

Other advantages of the SoF are that it is an environmentally friendly technique, requiring less solvent than the standard SoL technique and no cooling water or heating (*1, 18*). As extractions do not require refluxing of solvents or azeotropic mixtures, a wider range of solvent mixtures can be employed in the SoF than the SoL technique, including aqueous solvent mixtures. This will facilitate the optimization of extraction conditions for other natural plant products. Formation of artifacts will be minimal as extractions are carried out at room temperature; this will also be useful for the extraction of plant phenolics (*26*). Recent experiments (unpublished) indicated that SoF can be used for the extraction of flavonoids from plant materials. SoF is, therefore, a potentially useful technique for the rapid preparation of plant extracts for screening of phytochemicals or for bioassay studies.

Safety. SoF extractions are performed in an enclosed system and do not require any special precautions.

ABBREVIATIONS USED

HEM, hexane−ethyl acetate−methanol; CMW, chloroform−methanol−water; GC−MS, gas chromatography−mass spectrometry; rt, room temperature; SoF, Soxflo; SoL, Soxhlet; TLC, thin-layer chromatography.

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