Purification of the Alkaloid Lycorine and Simultaneous Analysis of Ascorbic Acid and Lycorine by Micellar Electrokinetic Capillary Chromatography¹

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The pyrrollophenanthridine alkaloid lycorine has frequently been used as a specific inhibitor to help elucidate the function of ascorbic acid (vitamin C) in a wide range of biological processes. It was recently reported that this function can be exercised by inhibiting the activity of L-galactono-1,4-lactone dehydrogenase, the terminal enzyme of ascorbic acid biosynthesis, although working with the purified enzyme, we have been unable to repeat this result. Here, we present a procedure for the purification and analysis of lycorine by high-performance liquid chromatography from two *Crinum* species and describe for the first time a method that allows the simultaneous analysis of ascorbic acid and lycorine in tissue extracts of *Crinum asiaticum* by micellar electrokinetic chromatography.

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Lycorine (2,4,5,7,12b,12c-hexahydro-1H-[1,3]dioxolo-[4,5-j]pyrolo[3,2,1-de]phenanthridine-1,2-diol) is an alkaloid present in various Amaryllidaceae (1) which has been reported to be able to inhibit ascorbic acid (L-AA,³ vitamin C) biosynthesis (increasing dehydroascorbic acid levels), at concentrations as low as 1 μ M (2, 3). It

was recently reported that this inhibition occurs by interaction of the alkaloid with galactono-1,4-lactone dehydrogenase, the terminal enzyme in one of the proposed pathways of L-AA biosynthesis in plants (4–6), although work in our own laboratory using the purified enzyme has failed to confirm this (7). It is also well documented that lycorine is a powerful inhibitor of growth and cell division in higher plants, algae, and yeasts (2, 8–10); that it interferes with the peptidyl-transferase center on ribosomes (11); and that it has antimalarial activities (12) as well as antitumor activities (1). The effects of lycorine on L-AA biosynthesis have been reported to occur at concentrations below those at which protein synthesis is effected (2, 13), but it seems difficult to completely rule out nonspecific effects of this alkaloid since it has been reported that, at least in yeasts, lycorine is able to interact directly with mitochondrial DNA (14–18).

L-AA plays a pivotal role in a number of important biological processes, including the biosynthesis of hydroxyproline-rich proteins (10, 13) and the scavenging of oxygen radicals generated during photosynthesis and under conditions of biotic and abiotic stress (19–22). As such, it is involved in the mediation of systemic acquired resistance following the hypersensitive response (23, 24) and, further, L-AA has been implicated as being involved in the regulation of cell growth (2, 10) and cell elongation (25–28). This means that lycorine is a potentially important tool for the manipulation of L-AA levels during investigations into any of these processes. Despite this, there are few protocols specifically available for the isolation of lycorine. Methods for the identification of the “total alkaloids” fraction of a tissue typically require extraction with alcohol fol-

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³ Abbreviations used: BGE, background electrolyte; L-AA, L-ascorbic acid; MPA, metaphosphoric acid; MEKC, micellar electrokinetic chromatography; SO₄, H₂SO₄; TLC, thin-layer chromatography.

lowed by a series of partitionings with solvents of differing polarities and then recrystallization (12, 29, 30) and/or the use of low-pressure column techniques (31, 32). In all cases, while the procedures are simple to execute, they are labor-intensive and are not suited to the routine isolation of milligram quantities of lycorine, because of the large number of handling steps.

As part of our studies on the role of L-AA during plant stress responses, we were interested in evaluating the use of lycorine as a tool to manipulate L-AA levels. Here, we present a protocol that allows the rapid purification of lycorine by preparative HPLC with a minimum number of pretreatment steps. In addition to semipreparative and analytical HPLC procedures for the analysis of lycorine, we describe for the first time a micellar electrokinetic capillary chromatography (MEKC) protocol suitable for the simultaneous quantitation of lycorine and L-AA in leaf and root extracts of tissues of *Crinum asiaticum*.

EXPERIMENTAL

Reagents

Ethylenediaminetetraacetic acid.2Na (EDTA.2Na), Tris base, and sodium hydroxide were purchased from Acros (Beerse, Belgium); L-AA was from Aldrich Chemie (Milwaukee, WI); and HCl was from Merck (Darmstadt, Germany). HPLC-grade methanol and HPLC-grade acetonitrile were obtained from Lab Scan (Dublin, Ireland). 5g "High Load" C₁₈ SPE cartridges were from Alltech (Deerfield, IL). Aluminum-backed silica gel thin-layer chromatography (TLC) plates were obtained from Merck. Fused silica capillaries (60 cm × 50 μm i.d., 363 μm o.d.) were purchased from Waters Associates (Milford, MA) and cut according to requirements. Lycorine hydrochloride was obtained from Molecular Probes (Eugene, OR).

Preparative Extraction

Nonflowering, whole plants from *Crinum jagus* or *C. asiaticum* var. *Japonicum* were harvested and extracted the same day essentially as follows: Plants were washed in distilled water and blotted dry and the leaves, stem, and root material were separated and weighed. Tissues were extracted by blending in 1.5% sulfuric acid at 4°C. The residue was filtered through three layers of glass fiber cloth (Miracloth; Calbiochem, La Jolla, CA) and reextracted up to three times more with 1% sulfuric acid. The alkaloid content at each stage of the extraction was determined by isocratic HPLC. The filtrate was partitioned once against an equal volume of *n*-heptane by vortexing for ≈1 min. The phases were partitioned out by centrifugation at 3304g in an HB-4 Sorvall (DuPont Medical Products, Wilmington, DE) swing-out rotor for 10 min and the

n-heptane upper phase was discarded. Fractions were kept at 4°C for further processing or stored frozen at −18°C. Aliquots of 50 ml were then passed through a Whatman GF/C glass fiber prefilter under positive pressure. The filtrate was made 5% (v/v) with acetonitrile and the entire 50 ml was loaded onto a 5g C₁₈ SPE cartridge, preconditioned with methanol and 1% H₂SO₄ containing 5% (v/v) acetonitrile. The cartridge was washed with a further 3 bed volumes of 1% H₂SO₄/5% acetonitrile to quantitatively recover all lycorine present (as judged by HPLC and TLC) in the flowthrough. Cartridges were discarded after a single use.

HPLC

Analytical HPLC was carried out using a Waters 600E pump and controller with a Waters 997 photodiode array detector. Data were collected over the range 200–350 nm at a frequency of 2 Hz and a spectral resolution of 1.2 nm. Quantitation was carried out at 292 or 210 nm. The instruments were controlled and the data collected and analyzed using the Millennium 2010 Chromatography Management System (version 2.15; Waters Associates). Injections of 5–10 μl were made manually using a Rheodyne injector and a 20-μl injection loop onto a C₁₈, 3-μm spherical particle size, 150 × 4.6-mm i.d., reversed-phase HPLC column (Bio-Rad, Hercules, CA). Preparative separations were carried out using a Varian Model 5500 HPLC instrument with multiple injections of 1 ml onto a 10 × 450-mm preparative HPLC column filled with 10-μm C₁₈ packing material. Preparative separations were carried out at 7 ml/min with a 12-min linear gradient of 0–100% acetonitrile in 10 mM ammonium carbonate (pH 9.3). Analytical separations were carried out at 0.8 ml/min using a 15-min linear gradient of 10 mM ammonium carbonate (pH 9.3) to 100% acetonitrile/10 mM ammonium carbonate, 90/10 (v/v), or isocratically on the same column with ammonium carbonate–acetonitrile (pH 9.3), 53:47 (v/v). The HPLC analysis of L-AA was carried out essentially as previously described (33).

TLC

Sample purity and the progress of the extraction procedures were also monitored by silica gel TLC. Here, 10-μl samples were dried down under a stream of warm air onto aluminum-backed silica gel TLC plates (Merck). These plates were developed in filter-paper-lined glass tanks using a freshly prepared mixture of chloroform–methanol, 2:1 (v/v), containing 2% (v/v) acetic acid. Fractions were visualized by spraying with a 10% ethanolic solution of H₂SO₄ and heating at ≈110°C for 10–15 min. Lycorine-containing fractions were visualized as yellowish spots with *R_f* values of ≈0.4.

Electrospray Mass Spectrometry

HPLC-purified lycorine fractions were dried under vacuum and redissolved in 0.1% trifluoroacetic acid only immediately prior to analysis, since lycorine has been reported to be unstable in trifluoroacetic acid (34). Manual injections were made on a Fisons VG platform with a cone voltage of 15 or 25 V. Data were collected and analyzed using the Fisons software package "Mass Lynx" (scan range 50–350/500).

HPCE

Alkaloid preparations and plant extracts dissolved in 1.5% H_2SO_4 /1 mM EDTA or 3% metaphosphoric acid/1 mM EDTA (MPA/EDTA) were analyzed on either a Waters Quanta 4000 capillary electrophoresis unit with fixed-wavelength ultraviolet (UV) detection at 185, 214, or 254 nm or a Spectrophoresis Ultra CE unit (Thermo Separations Products, San Jose, CA) with a Model 3000 UV detector, with scanning and multiple wavelength UV-detection possibilities. In both cases, samples were separated on a 34 cm to detector (41 cm total length) $\times$ 50- μm fused-silica capillary with 200 mM borate (pH 9.0)/50 mM SDS as background electrolyte (BGE) and an applied voltage of +20 kV. Injections were performed hydrostatically for up to 40 s with the Quanta 4000 and hydrodynamically for up to 5 s at a pressure of 0.8 p.s.i. (5516 Pa) with the Spectrophoresis Ultra unit. A 0.01% solution of formamide in BGE was used as an electroosmotic flow marker. The capillary was regenerated between analyses by rinsing with 200 mM borate (pH 9.0)/200 mM SDS, 1:1 (v/v), followed by BGE. Data were collected at a rate of 12 Hz and analyzed using a computer running the proprietary software, i.e., Maxima 820, version 3.31 (Quanta 4000), or PC1000, version 3.35 (Spectrophoresis Ultra). Simultaneous, fixed-wavelength determination of L-AA and lycorine was carried at 185 nm (Quanta 4000) or by dual-wavelength detection at 202 and 267 nm (Spectrophoresis Ultra).

Extraction of Plant Tissues for Simultaneous L-AA and Lycorine Analysis

Aliquots of 50–300 mg of plant tissue were accurately weighed and snap frozen in liquid nitrogen. Samples were crushed in liquid nitrogen using a pre-cooled pestle and mortar before addition of 0.4 ml extraction solvent (MPA/EDTA). The sample with frozen buffer was thoroughly homogenized and allowed to stand until the mixture thawed, whereupon it was transferred to a 2.0-ml Eppendorf tube with additional aliquots of extraction solvent. After centrifugation at 14,000 rpm (20,800 rcf) for 7 min, the extraction volume was accurately weighed by transfer to a 2.0-ml Eppendorf tube. The pellet was resuspended twice

more in fresh extraction buffer. Each time the supernatant was collected, weighed, and combined with the previous extract(s). The total extract was kept at 4°C in the dark prior to analysis or stored frozen at –20 or –70°C prior to HPLC and/or MEKC analysis.

Molar Extinction Coefficients

Molar extinction coefficients for purified lycorine dissolved in methanol were determined on an HP 8452A diode array spectrophotometer operated by a computer running the HP 89531A UV/Vis software.

Nuclear Magnetic Resonance

HPLC-purified lycorine fractions were dried, desiccated overnight, and redissolved in 0.4 ml d_6 -dimethyl sulfoxide, a saturated solution containing ≈ 0.1 mg of material. ^1H and ^{13}C NMR data were recorded on a Bruker WH-360 NMR spectrometer operating at 360.01 MHz for the ^1H NMR spectra and at 90.556 MHz for the ^{13}C NMR spectra using pulse angles of 19 and 18°, respectively.

RESULTS AND DISCUSSION

Preparative Extraction of Lycorine

We found that by using 1% H_2SO_4 as extraction solvent, the only additional treatment required prior to semipreparative HPLC was to partition extracts once against an equal volume of *n*-heptane and then to pass aliquots of the extract [made 5% (v/v) with acetonitrile] through a C_{18} SPE cartridge to remove pigmented, hydrophobic contaminants. Lycorine was quantitatively recovered in the flowthrough, as determined by TLC and HPLC analysis of aliquots of the eluant.

As previously reported (35), the quantities of lycorine present varied markedly depending on the type of tissue analyzed. In the whole plant, after preparative extraction of *C. asiaticum* (Table 1), the largest amounts were located in the roots. A cumulative plot of lycorine recovered versus milliliters of extraction solvent is logarithmic and indicates that our extraction was incomplete, but extrapolation of the data to 100% extraction generates values for the total lycorine content of the roots, stem, and leaves of 8.3, 2.5, and 2.0 $\mu\text{mol/g}$ fresh wt, respectively. From these figures, we calculate that we recovered 93, 80, and 77% of the lycorine present in the respective samples. These tissue concentrations are higher than the values later obtained for the quantitative analysis of portions of a single leaf and root (harvested later in the year from a different plant), suggesting that there can be considerable intra- and interplant variation in alkaloid levels depending on the season, tissue, and developmental stage. Results for the preparative whole-plant extraction are summarized in Table 1.

TABLE 1
Extraction of Lycorine from *C. asiaticum*

Sample	Fresh weight (g)	Extraction number	Extraction volume (ml)	Lycorine recovered (μg/g fresh wt)
Roots	1353	1	1075	1630
		2	380	289
		3	330	188
		4	180	74
		Total mg extracted: 2950		
Stem	1371	1	930	435
		2	216	56
		3	265	55
		4	215	30
		Total mg extracted: 798		
Leaves	1220	1	520	201
		2	385	117
		3	250	65
		4	270	57
		Total mg extracted: 535		

Note. Yields of lycorine were calculated after HPLC analysis of extracts and using the standard curves determined in the following section, i.e., $y = 1.197 \times 10^7 x - 4.113 \times 10^3$, $r = 0.9998$, $n = 21$. Each point represents the mean of three injections.

HPLC Analysis of Lycorine

A number of alternative separation schemes were examined including normal-phase HPLC, and isocratic and gradient reversed-phase separations under both acidic and basic conditions. However, the most satisfactory separations were obtained using the basic mobile phase reported in (35). This volatile buffer system provided better peak profiles than other mobile phases, such as the methanol–water systems used by (30), and when used in a gradient system produced higher selectivity. Under semipreparative conditions, the lycorine eluted as a single peak with a retention time of 17 min, free of other contaminating peaks. The purity of the isolated peaks was confirmed by drying down and re-analyzing by TLC, by analytical gradient C_{18} reversed-phase HPLC, and by MEKC. All of these techniques demonstrated that the purified product was at least 98% pure after a single run. Purified lycorine, dissolved in water, phosphate (pH 7.3), or extraction solvent (MPA/EDTA), coeluted with authentic lycorine standard following MEKC and gradient analytical HPLC and had an identical absorption spectrum (190–350 nm). The HPLC-purified lycorine was used to construct standard curves to allow direct quantitation by HPLC of extracts. With these, linearity of detector response at 292 nm (the absorption maximum) was observed over a concentration range of at least 0.02–1 mg/ml (70 μM –350 mM) or 5–290 μg (17–1000 nmol) injected.

Molar Extinction Coefficients

Using HPLC-purified lycorine, we established molar absorption coefficients of 13,440 (210 nm), 3660 (234 nm),

and 5190 (292 nm). These correspond closely to the extinction coefficients reported by Likhitwitayawuid *et al.* (12) of 3529 (240 nm) and 5017 (290 nm).

Electrospray Mass Spectrometry

The molecular ion was observed at a m/e of 287.9 (expected 287.3). Only one other significant peak was found at m/e 240.8. We did not observe any higher molecular weight species that might be indicative of glycosylated or phosphatidylated derivatives of lycorine reportedly present in the fruit, flowers, and sap of various *Crinum* species (1, 30, 36).

NMR

The ^{13}C NMR chemical shifts obtained were found to be in full agreement with previously published data (12, 29). The ^1H NMR spectra, however, are difficult to analyze without thorough two-dimensional experiments and, moreover, as there are so many overlaps of resonances, second-order effects may make the spectra impossible to decipher. Nonetheless, the data that can be extracted with certainty agree fully with the published data referred to above.

Simultaneous Extraction of Lycorine and Ascorbic Acid

Because of the reported influence of lycorine on L-AA biosynthesis (2, 4–6, 10), we were interested in developing a system to allow the simultaneous analysis of both lycorine and L-AA from a single tissue extract. In the quantitation of L-AA however, partic-

FIG. 1. Comparison of the recoveries of lycorine (A) and L-AA (B) from root and leaf tissues of *C. asiaticum* using either 3% MPA/1 mM EDTA (MPA/EDTA) or 1.5% SO₄/1 mM EDTA (SO₄/EDTA) as extraction solvents. Lycorine values were determined by isocratic HPLC (see Experimental). Using MPA/EDTA, 87.7 mg of leaf and 364.1 mg root tissue were extracted, and using SO₄/EDTA, 118.7 mg of leaf and 333.6 mg of root tissue. Total extraction volumes were $\approx$ 2.5 ml after four separate extractions. L-AA values were derived by isocratic HPLC as described in the text.

ular care must be taken during sample preparation and storage to prevent oxidation of the L-AA [see, e.g., (37)]. One of the most critical factors influencing the stability of L-AA is the choice of extraction solvent used and we, like others (38, 39), found that MPA/EDTA both stabilizes L-AA and has the highest extraction yields for L-AA. To confirm the suitability of MPA as a solvent for lycorine, we made a quantitative comparison of the effectiveness of the two solvents, MPA/EDTA and 1.5% H₂SO₄/1 mM EDTA (SO₄/EDTA), in extracting lycorine and L-AA from portions of the same leaf and root samples of *C.*

asiaticum. Samples were processed in parallel and results are summarized in Fig. 1.

As can be seen, extraction of aliquots of the same root and leaf tissues using either MPA/EDTA or SO₄/EDTA produced essentially identical results for the lycorine content. The proportion of extraction solvent:tissue extracted used is clearly important and quantitative yields were obtained from root or leaf tissue when the ratio of solvent:tissue was greater than at least 4:1 μ l/mg fresh wt. The smaller amount of leaf tissue extracted explains why only two extraction cycles were required to recover all lycorine from these samples. In

FIG. 2. (A) Influence of concentration of SDS in 200 mM borate (pH 9.0) on the relative migration times of L-AA and lycorine. (B). Influence of the proportion of acetonitrile in 100 mM borate (pH 9.0)/50 mM SDS on the relative migration times of L-AA and lycorine.

principle however, a minimum of two extractions will always be required to recover analytes retained in the extraction solvent contained within the pellet following the first extraction.

As expected, however, yields of L-AA were severely depressed when SO₄/EDTA was used as extraction solvent (Fig. 1B). This is presumably due either to oxidation during the extraction process or to incomplete inactivation of L-AA-metabolizing enzymes. These results were later confirmed using the optimized MEKC separation. In addition to the poor recoveries of L-AA though, there were also clear differences between the electropherograms of either MPA/EDTA or SO₄/

EDTA extracts, with peaks being generally broader and less well resolved in SO₄/EDTA.

Total yields of lycorine obtained with an MPA/EDTA extraction were 4370 nmol/g fresh wt in leaf and 1060 nmol/g fresh wt in root. The corresponding values for the SO₄/EDTA extraction were 4090 nmol/g fresh wt (leaf) and 1050 nmol/g fresh wt (root). Total yields of L-AA using MPA/EDTA were 871 and 630 nmol/g fresh wt for leaf and root, respectively, whereas the corresponding values for the SO₄/EDTA extracts were 563 and 0 nmol/g fresh wt. These results clearly demonstrate the unsuitability of SO₄/EDTA as extraction solvent for this dual analysis.

FIG. 3. Typical electropherogram obtained for the simultaneous MEKC analysis of lycorine and L-AA from MPA/EDTA extracts of an 82-mg aliquot of leaf tissue from *C. asiaticum*. Samples were injected for 5 s at 0.8 p.s.i. and separated on a 34 cm (to detector) $\times$ 50- μ m fused-silica capillary thermostated at 30°C, using 200 mM borate (pH 9.0)/50 mM SDS as BGE at +20 kV. Detection was carried out at the UV-absorption maxima which at this pH are 202 and 267 nm for lycorine and L-AA, respectively. Data were acquired at a rate of 12 Hz.

In a separate experiment to determine the reproducibility of the extraction procedure, replicate samples of *C. asiaticum* leaf tissue were extracted with MPA/EDTA and the levels of L-AA and lycorine were quantitated by MEKC. For three separate extractions, each analyzed twice, values for L-AA determined at 267 nm were 2520 nmol/g fresh wt $\pm$ 4.7% and for lycorine 582 nmol/g fresh wt $\pm$ 4.2% as determined at 202 nm.

Optimization of MEKC Separations

In an acidic BGE [100 mM phosphate, pH 5.0, 5.8, 6.5; 50–100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.14], purified lycorine was found to migrate as an asymmetric peak ahead of the neutral electroosmotic flow marker (formamide), while under basic conditions (100 mM borate, pH 9.0) it comigrated along with formamide. In all cases, there was little selectivity displayed in the separation. We had already developed a system for the HPCE analysis of L-AA in MPA extracts of plant tissues using 200 mM borate (pH 9.0) as BGE

(33, 40), but to obtain resolution of both the alkaloid and L-AA together it was necessary to add SDS. The inclusion of SDS has a dramatic effect on the relative migration times of lycorine, presumably via interaction between the anionic SDS micelles and the cationic ring nitrogen of lycorine, but SDS has relatively little influence on the migration of L-AA. Resolutions of biological extracts could therefore easily be tailored by manipulation of the SDS concentrations. These results, along with the effects of acetonitrile, are summarized in Fig. 2. The UV-absorption maxima of lycorine and L-AA are approximately 202 and 267 nm, respectively, at this pH, but for simultaneous analysis of these two components using fixed-wavelength detection (Quanta 4000), UV detection was carried out at 185 nm because lycorine has a UV-absorption minimum around 260 nm.

From these results, optimal separations were standardized as 200 mM borate (pH 9.0)/50 mM SDS on a 34 cm (to detector) $\times$ 50- μ m fused-silica capillary with an applied voltage of +20 kV. A typical electrophero-

gram for the analysis of purified L-AA and lycorine from extracts of *C. asiaticum* leaf tissue is shown in Fig. 3.

Detector response was linear between at least 17 and 700 μM for lycorine and between at least 56 and 5600 μM for L-AA. Minimum detection limits at 185 nm (Quanta 4000), set at approximately threefold baseline noise, were in the region of 17 and 14 μM for lycorine and L-AA, respectively. Monitoring at the UV-absorption maxima of these compounds however increased the sensitivity of detection so that minimum detection limits were in the region of 1 μM for lycorine at 202 nm and 2.5 μM for L-AA at 267 nm.

For a standard solution of lycorine in MPA/EDTA, absolute migration times for a triplicate analysis agreed within $\pm 0.2\%$, and the time-corrected areas agreed within $\pm 3\%$. Errors tended to be slightly greater for the analysis of biological extracts with the reproducibility between migration times varying from ≈ 0.2 to 2% and the time-corrected areas from ≈ 1.3 to 6%.

Biological Activity/Applications

Surprisingly, we have been unable to demonstrate the reported biological effects of lycorine on L-AA biosynthesis (2–6) using photo-heterotrophic *Arabidopsis thaliana* cell suspension cultures or to reproduce the reported inhibition of galactono-1,4-lactone dehydrogenase with concentrations of lycorine as high as 200 μM (7). We have at present no explanation for these results, which are currently under further investigation.

CONCLUSIONS

The preparative procedures described here provide a simple means for the purification of milligram amounts of lycorine suitable for use in biological studies. The material obtained after HPLC is at least 98% pure as judged by analytical HPLC and MEKC; it has been characterized by ^{13}C and ^1H NMR and by electrospray mass spectrometry and it comigrates in all separation systems described (see Experimental) with the commercially available standard. We further describe for the first time a MEKC method for the simultaneous quantitation of L-AA and lycorine in plant tissue extracts allowing direct determination of the influence of lycorine on L-AA levels *in vivo*. Such a separation is not possible by liquid chromatography because of the differences in the physicochemical properties of the two compounds, although there are suitable procedures for the analysis of either component independently (29, 32, 34, 37, 38). With simple, uncoated fused-silica capillaries however, the two compounds can be analyzed within 6 min using MEKC. Whereas the concentration limits of detection are higher for MEKC than HPLC, the MEKC approach has a number of advantages, nota-

bly the speed of analysis, the small volumes of material required, the short turnaround time between analyses, and the fact that both components can be simultaneously analyzed from a single extract.

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