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Solid-Phase Extraction of Ascorbic Acid 2-Sulfate from Cysts of the Brine Shrimp *Artemia franciscana*

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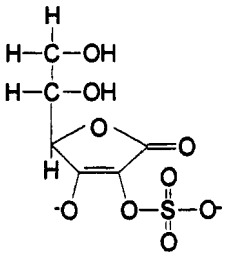
Two procedures are described for the solid-phase extraction of ascorbic acid 2-sulfate from cysts of the brine shrimp *Artemia*. The first one is based on reversed-phase ion pair extraction on an octadecylsilica cartridge using tetrabutylammonium as the counterion, followed by elution with methanol. In the second procedure ascorbic acid 2-sulfate is retained on a DEAE silica cartridge and eluted with 0.1 M sodium salicylate. Both approaches were used as part of a quantitative reversed-phase ion pair liquid chromatographic method with detection at 254 nm. The anion exchange extraction procedure is superior to its ion pair counterpart in terms of recovery (88.7 ± 3.5% versus 63.0 ± 12.7%), reproducibility (CVs of 3.6–7.7% versus 6.3–18.7%), and simplicity.

Ascorbic acid 2-sulfate (**I**; AAS) has been reported as a metabolite of ascorbic acid in humans[1] and several animal species.[2] Particularly intriguing is the biochemical significance



I: Ascorbic acid-2-sulfate

of the accumulation of AAS in cysts of the brine shrimp *Artemia*, from which the compound was first isolated in 1969.[3] Whether AAS serves as a storage form of ascorbic acid to satisfy larval requirements[3] after hatching or acts as a sulfating agent during embryonic development[3,4] has not been unequivocally ascertained. In aquaculture fish feed is commonly supplemented with AAS as a stable source of vitamin C,[5] although its bioavailability is questionable.[6,7] The existing spectrophotometric[8,9] and liquid chromatographic[10–13] methods for the determination of AAS in brine shrimp, fish, and fish feeds suffer from poor selectivity and sensitivity, mainly because of insufficient sample pretreatment. However, the high polarity and water solubility of AAS do not make a purification and concentration of aqueous extracts straightforward. The compound does not partition in organic solvents, and problems of retention or elution may have precluded the development of solid-phase extraction (SPE) methods so far. This lack of effective sample preparation is not counterbalanced by the final detection itself because AAS absorbs at a nonselective wavelength (232–254 nm, depending on the pH),[2] cannot be directly converted to a colored derivative, and is oxidized electrochemically at a high potential (>0.9 V).[12] Therefore, in some liquid chromatographic procedures[12] as well as in the spectrophotometric ones,[8,9] the compound is quantitated indirectly after its conversion to the more readily detectable ascorbic acid.

This is the first report on the use of solid-phase extraction on microparticulate cartridges as a sample preparation for the determination of AAS in *Artemia* cysts. Of the two approaches that were tested, i.e., reversed-phase ion pair and anion exchange extraction, the latter proved superior in terms of recovery, reproducibility, and simplicity.

EXPERIMENTAL SECTION

Chemicals and Reagents. Methanol (Analar grade) and acetonitrile (HPLC grade) were purchased from BDH (Poole, UK) and ROMIL (Loughborough, UK), respectively. Dipotassium ascorbic acid 2-sulfate came from Sigma (St. Louis, MO). Stock solutions were prepared in distilled water. Isoascorbic acid 2-sulfate (mixture of stereomers) was kindly donated by A. Crevat (Marseille, France). Tetrabutylammonium phosphate low-UV reagent (PIC A) was obtained from Millipore (Bedford, MA). Bond Elut C_{18} (500 mg, 3 mL), Bond Elut PRS (500 mg, 3 mL) and Bond Elut DEAE (100 mg, 1 mL) cartridges were from Varian Preparation Products (Harbor City, CA). These minicolumns are packed with a 40-μm derivatized silica containing octadecyl (C_{18}), propylsulfonic acid (PRS), and diethylaminopropyl (DEAE) bonded phases, respectively. All other chemicals (analytical

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(1) Baker, E. M.; Hammer, D. C.; March, S. C.; Tolbert, B. M.; Canham, J. E. *Science* **1971**, *173*, 826–827.
(2) Tolbert, B. M.; Downing, M.; Carlson, R. W.; Knight, M. K.; Baker, E. M. *Ann. N.Y. Acad. Sci.* **1975**, *258*, 48–69.
(3) Mead, C. G.; Finamore, F. J. *Biochemistry* **1969**, *8*, 2652–2655.
(4) Bond, A. D.; McClelland, B. W.; Einstein, J. R.; Finamore, F. J. *Arch. Biochem. Biophys.* **1972**, *153*, 207–214.
(5) Soliman, A. K.; Jauncey, K.; Roberts, R. J. *Aquaculture* **1986**, 52, 1–10.
(6) Dabrowski, K.; El-Fiky, N.; Köck, G.; Frigg, M.; Wieser, W. *Aquaculture* **1990**, *91*, 317–337.
(7) Dabrowski, K. *J. Comp. Physiol. B* **1990**, *160*, 549–561.
(8) Dabrowski, K.; Hinterleitner, S. *Analyst* **1989**, *114*, 83–87.
(9) Dabrowski, K.; Lackner, R.; Doblander, C. *Can. J. Fish. Aquat. Sci.* **1990**, *47*, 1518–1525.
(10) Felton, S. P.; Halver, J. E. *Aquacult. Fish. Manage* **1987**, *18*, 387–390.
(11) Schüep, W.; Marmet, J.; Studer, W. *Aquaculture* **1989**, *79*, 249–258.
(12) Wang, X. Y.; Seib, P. A. *Aquaculture* **1990**, *87*, 65–84.
(13) Hoffman, K. M.; Brown, P. R.; Maugle, P. D. *J. Liq. Chromatogr.* **1992**, *15*, 2581–2610.

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grade or ACS grade) were purchased from Aldrich (Milwaukee, WI), Sigma, and Merck (Darmstadt, Germany).

Biological Samples. *Artemia* cysts came from the collection at the Artemia Reference Center (Gent, Belgium). For the method evaluation, either whole cysts from the San Francisco Bay strain (batch 1090) (California) or decapsulated cysts were used. Developmental experiments were carried out using decapsulated[14] cysts (Great Salt Lake, UT) obtained from Artemia Systems NV (Baasrode, Belgium). Sea bass larvae (45 days old) were purchased from the commercial hatchery SEPIA (Gravelines, France).

Apparatus. The HPLC system consisted of an LKB 2150 pump (LKB, Bromma, Sweden), an N-60 valve injector fitted with a 50-100-μL loop (Valco, Houston, TX), a Kontron 430 UV–Vis absorbance detector (Kontron, Milan, Italy), and a Philips PM 8252A strip chart recorder (Pye Unicam, Cambridge, UK). For the on-line recording of absorption spectra of chromatographic peaks, an HP 1040A photodiode array detector was used, coupled with an HP 85 computer, an HP 82901M flexible disk drive, and an HP 7470A graphics plotter (all from Hewlett Packard, Palo Alto, CA).

Chromatographic Conditions. Two reversed-phase ion pair HPLC systems were developed. In the first one, a 5-μm Nucleosil C_{18} column (Macherey and Nagel, Düren, Germany) was used and the mobile phase was a mixture of acetonitrile (4–6 parts) and a pH 5.0 60 mM acetate buffer (94–96 parts) containing 1.5 mM dimethylhexylamine. The second system consisted of a 15 × 0.39 cm 4-μm Waters Novapak C_{18} column (Millipore), eluted with a 1:99 (v/v) mixture of acetonitrile–water, containing 48 mM potassium phosphate (pH 5.3), 1 mM PIC A, and 0.1% triethylamine. The flow rate was 1 mL/min, and detection was carried out at 254 nm.

Preparation of Crude Extracts of *Artemia* Cysts. Approximately 20 mg of dry *Artemia* cysts was homogenized in 2 mL of pH 5.0 60 mM acetate buffer using an all-glass Potter Elvehjem tube. After centrifugation (1500*g*, 5 min) the supernatant was transferred to a polypropylene tube and the same homogenization procedure was repeated once more on the residue. The final residue was rinsed twice with 0.5 mL of the acetate buffer, and all supernatants were combined.

Purification of Crude Extracts by Ion Pair Reversed-Phase Solid-Phase Extraction. The crude extract (5 mL) was mixed with 0.5 mL of 125 mM tetrabutylammonium hydrogen sulfate (TBAHS) and applied on top of a Bond Elut C_{18} cartridge which had been preconditioned with 2.5 mL of methanol and 2.5 mL of a mixture of pH 5.0 60 mM acetate buffer and TBAHS (5:0.5, v/v). After the cartridge was washed with the buffered TBAHS, the absorbed compounds were eluted with 5 mL of methanol. The methanolic extract was evaporated to dryness at room temperature and under vacuum using an Evapo-mix apparatus (Büchler Instruments, Fort Lee, NJ). The residue was reconstituted with 2 mL of water–methanol (94:6, v/v), and the mixture was filtered through a Bond Elut PRS cartridge, which had been preconditioned with 2.5 mL of methanol and 2.5 mL of methanol–water (94:6, v/v). The filtrate was collected, combined with a final rinse (1 mL of methanol–water, 94:6, v/v) of the cartridge, and adjusted to 5.0 mL in a volumetric flask with methanol–water (94:6, v/v). A 50-μL aliquot of this solution was injected.

Purification of Crude Extracts by Anion Exchange Chromatography. The crude extract (5 mL) was quantitatively transferred to a Bond Elut DEAE cartridge which had been preconditioned with methanol (1 mL), water (1 mL), and the pH 5.0 acetate buffer (1 mL). After the cartridge was washed with 1 mL of water, elution was carried out with 1 mL of 0.1 M sodium salicylate. The pH of the eluate was adjusted to 2.0 with 0.1 mL of 1 M HCl, and the solution was extracted twice with 5 mL of diethyl ether. The organic layer was removed and transferred to a waste bottle for storage until disposal by a specialized company. The aqueous solution was purged with nitrogen to remove the last traces of ether. The latter was carried out in a fume hood, but the small quantity of ether left on top of the aqueous phase after removal of the organic layer did not represent an appreciable hazard. Finally, the pH was readjusted to 5 with 1 M sodium hydroxide and the volume was brought to 5.0 mL in a volumetric flask. A 50-100-μL aliquot was injected.

Quantitation and Method Validation. Because *Artemia* cysts devoid of AAS or containing low concentrations of it were not available, linearity, recovery, and detection limit were evaluated by analyzing buffer solutions supplemented with the compound of interest. Standard curves were constructed by plotting absolute peak heights against the total quantities of AAS in the solution (5 mL). The quantities in the unknown extracts, corresponding to approximately 20 mg of dry weight, were calculated from the standard curve and extrapolated per gram of dry weight. Recovery was determined by comparing the peak heights obtained with and without (direct injection) SPE. The precision was determined by repeatedly analyzing cysts.

RESULTS AND DISCUSSION

Solid-Phase Extraction. Sample preparation in existing HPLC assays of AAS is limited to an aqueous extraction, usually with strong acid, followed by the direct injection of an aliquot of the crude extract on the column.[10,12,13] Depending on the complexity of the sample, matrix components will more or less interfere with the peak of AAS, particularly in connection with UV detection.[10,13] With electrochemical detection of AAS selectivity is only marginally better.[12] Furthermore, the repeated injection of crude aqueous extracts without cleanup is likely to cause progressive column deterioration and the presence of acid resulted in a distorted AAS peak in our chromatographic system. Hence, the routine applicability of current methods for AAS is questionable. The two SPE approaches that we developed for the purification of extracts take advantage of the anionic character of AAS. In the first procedure, an ion pair formed between AAS and TBAH is retained on a C_{18} bonded-phase cartridge. An attractive feature of this system is that the analyte is eluted with methanol so that the extract can be readily concentrated. Unfortunately, the remaining excess TBAH severely disturbed the chromatographic performance (peak shape and efficiency), so that extra cation exchange and dilution steps had to be included to eliminate this interference. The second SPE procedure employs anion exchange chromatography on DEAE

(14) Bruggeman, E.; Sorgeloos, P.; Vanhaecke, P. *The Brine Shrimp Artemia*; Universa Press: Wetteren, Belgium, 1980; pp 261–269.

Table 1. Effect of the Final Volume of the Extract on the Peak Height of AAS

vol (mL)	normalized[a] peak height (cm) extract of cysts	extract of std in buffer
1	5.6	9.2
2	10.1	16.8
3	12.2	18.9
4	13.1	18.5
5	13.4	19.3

[a] Corrected for detector sensitivity and the dilution factor.

silica, which is strongly retentive for AAS. The analyte could be readily eluted by high ionic strength acidic (pH 2) mixtures, including 0.1 M phosphate buffer and 0.1 M NaCl–0.01 M HCl. However, the injection of the eluate always caused a dramatic peak distortion, even after neutralization to pH 5. This was also true for 0.01 M sulfuric acid, which has been used to elute AAS from DEAE cellulose.[15] Removal of the sulfate with barium salts was not further considered because a substantial part (±50%) of the AAS coprecipitated. To reconcile chromatographic compatibility with AAS recovery, 0.1 M sodium salicylate was finally adopted as the eluent. Salicylate has a very strong affinity for anion exchange materials and can be removed easily from an acidified solution by extraction with diethyl ether. In the absence of such extraction, the AAS peak was once more distorted and a huge late-eluting peak, presumably due to salicylate itself, occurred in the chromatogram. An advantage of the strong interaction between AAS and the DEAE sorbent is that small-sized cartridges (100 mg) can be used, so that accordingly, elution with small volumes (1 mL) is feasible. However, even though the salicylate was exhaustively removed by extraction, some dilution of the final extract remained necessary to ensure optimal efficiency (as reflected by a maximal peak height) (Table 1).

Chromatography. The mobile phase of the first chromatographic system differed from that used by Tsao and Young[16] in its organic modifier (acetonitrile instead of ethanol) and the concentration of the dimethylhexylamine. Our initial work was carried out on Zorbax ODS, but the Nucleosil C_{18} column proved superior in terms of efficiency and peak shape. The second chromatographic system was based on a combination of two HPLC methods for the separation of adenine nucleotides[17,18] but was only used for qualitative purposes. Chromatograms of extracts of *Artemia* cysts obtained with the first system after reversed-phase ion pair and anion exchange SPE are shown in Figure 1.

Peak Identification. The AAS in extracts of *Artemia* cysts cochromatographed with authentic AAS in the two different chromatographic systems. Figure 2 shows a chromatogram obtained on the Novapak C_{18} column, used for peak confirmation. The second identity criterion for the AAS peak was the perfect match between its on-line-recorded absorption spectrum and that of synthetic AAS (not shown). No evidence

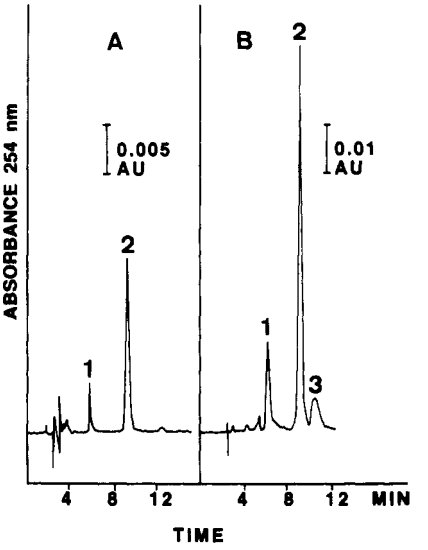

Figure 1. Chromatograms of extracts of dehydrated *Artemia* cysts after reversed-phase ion pair SPE (A) and after anion exchange SPE (B). Peak identification: 1, GMP; 2, AAS; 3, AMP. For chromatographic conditions, see the Experimental Section.

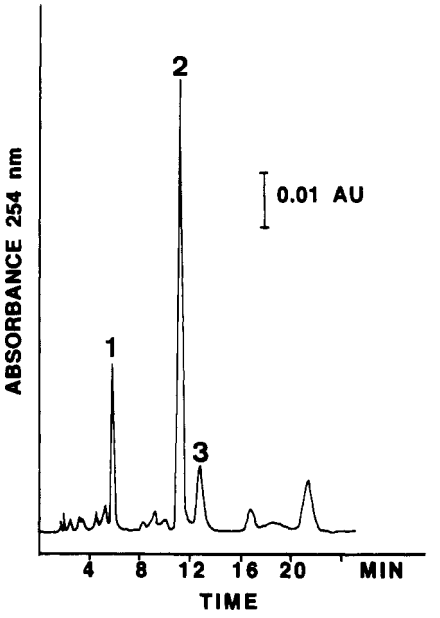

Figure 2. Chromatogram of an extract of dehydrated decapsulated cysts obtained on Novapak C18. For peak identities, see Figure 1.

was found of coeluting interferences, because the absorption spectra recorded at the up slope, apex, and down slope of the peak totally overlapped, indicating peak homogeneity. The accompanying peaks in the chromatograms obtained on dehydrated cysts (Figures 1 and 2) were GMP and AMP, products formed from the catabolism of (bis-$C_{5'}$-guanosyl) tetraphosphate (Gp_4G).[19] AMP levels rose dramatically in hydrated cysts (Figure 3), but neither AMP or GMP interfered with the quantitation of AAS.

Method Validation. Both methods were linear over the 2–43-μg range (total quantities in the sample) with correlation coefficients exceeding 0.999. A comparative evaluation of recovery and reproducibility for the two SPE procedures is presented in Tables 2 and 3. The superior performance of the

(15) March, S. C. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1972**, *31*, 705 (Abstr. 2762).
(16) Tsao, C. S.; Young, M. *J. Chromatogr.* **1985**, *330*, 408–411.
(17) Bodola, F.; Benedict, C. R. *J. Chromatogr.* **1988**, *459*, 281–289.
(18) Cordis, G. A.; Engelman, R. M.; Das, D. K. *J. Chromatogr.* **1988**, *459*, 229–236.
(19) Sillero, A.; Sillero, M. A. G. *Biochemistry and Cell Biology of Artemia*; CRC Press: Boca Raton, FL, 1989; pp 95–111.

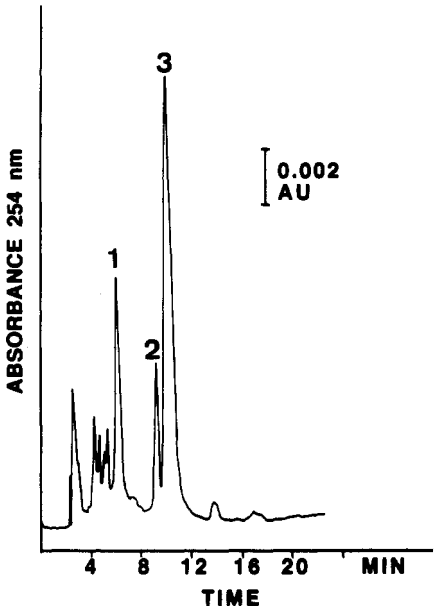

Figure 3. Chromatogram of an extract of hydrated (28 h of hydration) decapsulated *Artemia* cysts obtained on Nucleosil C18. For peak identities, see Figure 1.

Table 2. Recovery of AAS from Buffer Solutions

% rec	SD (%)	μg/sample	n
Method 1 (Reversed-Phase Ion Pair SPE)			
82.2	7.7	42	8
59.3	5.1	38	4
63.2	3.2	38	8
46.5	1.8	22.5	8
63.8	5.6	1.9	8
mean 63.0 ± 12.7			
Method 2 (Anion Exchange SPE)			
89.3	1.7	1.9	4
89.9	2.6	4.6	5
85.4	1.9	18.5	5
92.4	1.9	19	4
83.7	3.8	37	5
91.7	2.3	205	5
mean 88.7 ■ 3.5			

Table 3. Reproducibility data

$\bar{x}$ (μg/g)	SD (μg/g)	CV (%)	n
Method 1. Reversed-Phase SPE			
whole cysts			
760.2	29.8	3.9	8
826.0	154.3	18.7	8
Method 2. Anion exchange SPE			
whole cysts			
637.0	40.3	6.3	7
642.8	49.5	7.7	8
decapsulated cysts			
1077.6[a]	58.8	5.5	9
1193.5[a]	90.7	7.6	10
992.7[b]	35.8	3.6	9
620.8[c]	35.5	5.7	10

[a] Dehydrated. [b] Hydration for 8 h. [c] Hydration for 22 h.

anion exchange SPE is evident. A drawback of the ion pair SPE was its lower and more variable recovery. Yet within-run precision and linearity for this more complex approach were acceptable (one outlier accounted for the high CV value of 18.7%, Table 3). Overall reproducibility of the anion exchange approach was within 8% CV, even though no internal standard was used. No structural analog of AAS could be considered as a candidate for this purpose. Isoascorbic acid 2-sulfate, which is not readily available but has been synthesized by others,[20] coeluted with AAS. Adenosine 5′-sulfate was resolved from AAS but showed a different physicochemical behavior in terms of recovery, stability, and chromatographic properties (peak shape). The minimal detectable concentration of AAS in a solution obtained after anion exchange SPE was about 10 ng/mL, which would theoretically be equivalent to 3 μg/g of cysts, in case a 20-mg sample would be analyzed.

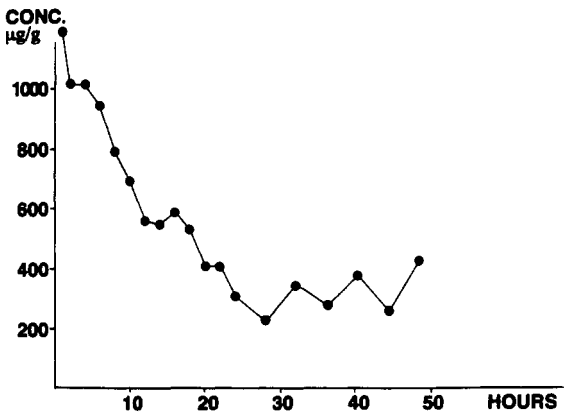

Figure 4. Disappearance of AAS in decapsulated *Artemia* cysts as a function of hydration time.

Applications. The above methods have been used routinely to study the fate of AAS in the course of *Artemia* embryonic development. Figure 4 illustrates the disappearance of AAS in *Artemia* cysts as a function of time after their hydration and the onset of embryonic development. These results corroborate the findings of Dabrowski[21] with respect to the conversion of AAS to free ascorbic acid during *Artemia* embryonic development. The simultaneous determination of ascorbic acid 2-sulfate and free ascorbic acid is not feasible because the latter is not recovered in either of the sample preparation procedures. With extracts of cysts, the extent of sample purification is sufficient to permit absorbance detection at 254 nm. When more complex matrices, e.g., fish and shrimp, were analyzed, electrochemical detection was required to eliminate some interfering peaks, in spite of the enhanced selectivity derived from the sample purification. Hence, at a later stage a slight modification of this method could also be considered for the determination of AAS in other biological materials.

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(20) Gallice, P. M.; Monti, J. P.; Braguer, D. L.; Monti, H. M.; Elsen, R. E.; Berland, Y. F.; Crevat, A. D. *Clin. Chem.* **1990**, *36*, 1369–1372.
(21) Dabrowski, K. *Can. J. Fish. Aquat. Sci.* **1991**, *48*, 1–4.

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