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Journal of Chromatography A, 1077 (2005) 37–43
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of [*S*,*S*′]-ethylenediamine disuccinic acid (EDDS) by high performance liquid chromatography after derivatization with FMOC

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Received 22 December 2004; received in revised form 8 April 2005; accepted 12 April 2005

Abstract

The paper describes a new HPLC method for the determination of ethylenediamine disuccinic acid (EDDS). EDDS is derivatized with FMOC reagent followed by HPLC separation on a reversed-phase column. The eluents consist of phosphate buffer at pH 6.8 and acetonitrile. Separation was carried out using gradient elution. The FMOC-EDDS derivative is detected with a fluorescence detector with an excitation wavelength of 265 nm and an emission wavelength of 313 nm. The detection limit is 0.01 μM. The method is applicable to the determination of the compound in water, soil solution and plant material at trace levels.

Keywords: Ethylenediamine disuccinic acid; HPLC; Fluorescence detection; EDDS; Mass spectrometric identification

1. Introduction

S,*S*-ethylenediamine-*N*,*N*′-disuccinic acid (EDDS) is a naturally occurring, biodegradable complexing agent [1]. Recently it has been used commercially in detergents [2,3] to replace EDTA, which is found to be too recalcitrant in the environment [4]. There has also been some interest in it for remediation of metal contaminated soils, both by soil washing and chelant enhanced phytoextraction [5–8].

The colorimetric detection of EDDS has been described but this method has a very high detection limit (0.1 mM) [1]. Knepper [3] mentions that the GC-based ISO method for complexing agents [9] can be used for the analysis of EDDS but no such use has been documented. In three investigations HPLC methods have been used for the analysis of EDDS but the details given are not comprehensive [11–13]. Although the detection limits are not given, it seems from the data that they would be relatively high. These methods are based on the photometric detection of CuEDDS or Fe(III)EDDS complexes. A HPLC method based on the photometric detection of Fe(III)EDDS has been described in detail but it is not suitable for use with complex matrices at trace levels of EDDS [14]. One method has been described using IC–ICP–MS for the detection of metal–EDDS complexes [10]. This method is suitable for trace analysis in natural waters but requires the use of an ICP–MS for detection and is therefore not suitable for routine analysis.

The aim of this work was to develop a HPLC based analytical method for EDDS that is applicable to a broad range of sample types and has a detection limit suitable for analysis at sub-micromolar concentrations. To achieve this goal we chose a FMOC (9-fluorenyl-methyl chloroformate) derivatization followed by HPLC separation and fluorescence detection. Fluorescence detection has advantages over UV detection, in that it gives low detection limits and high sensitivity due to low interferences. FMOC is a standard reagent for the determination of amino and imino acids [15,16]. FMOC has also been used for the derivatization of aminophosphonates [17], aminopolyphosphonates [18] and for glyphosate and its degradation product aminomethylphosphonic acid [19,20].

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doi:10.1016/j.chroma.2005.04.064

2. Experimental

2.1. Reagents and chemicals

Water was obtained from a MilliQ system (Millipore). All chemicals were obtained from Merck (Switzerland) and were analytical grade if not otherwise specified. All solvents were LiChrosolv grade. *S,S*-EDDS was obtained from Procter & Gamble (Belgium). A 1 M borate buffer was prepared from boric acid adjusted with sodium hydroxide to pH 6.2. A 0.1 M EDTA buffer was prepared by dissolving Na_2H_2EDTA in water and adjusting the pH with NaOH to 8 or 11.5. The metals were used in their nitrate forms. The FMOC reagent was prepared by dissolving 155 mg of 9-fluorenylmethyl chloroformate (FMOC-chloride, puriss; Fluka, Switzerland) in 40 ml acetone to give a concentration of 15 mM. It is important to prepare the FMOC reagent freshly each time it is used.

2.2. Derivatization of EDDS

0.2 ml EDTA buffer (pH 11.5) was added to 0.8 ml of sample. This was heated for 3 h at 90 °C. After cooling, 1.0 ml of the FMOC reagent was added and the sample was allowed to react for 30 min at room temperature. Two millilitres of dichloromethane were then added, the sample was shaken, centrifuged and 50 μl of the aqueous layer injected into the HPLC.

2.3. HPLC

A Jasco high-performance liquid chromatograph (PU-980; Jasco, Japan) equipped with a fluorescence detector (821-FP or FP-2020), using 265 nm as excitation and 313 nm as emission wavelength, and an autosampler 851-AS were used. An injection volume of 50 μl was used. The HPLC separations were performed on a Lichrospher 100 RP-18, 5 μm column (Merck, 12.5 cm length, 4 mm diameter). Some preliminary work was carried out on a PLRP-S polymer reversed phase C18 column (Polymer Laboratories, 15 cm length, 4 mm diameter). The aqueous mobile phase consisted of 0.05 M NaH_2PO_4 / 0.05 M Na_2HPO_4 with a pH of 6.8. The following gradient elution was used: 0–6 min from 10% acetonitrile to 20%, 6–8 min from 20 to 80% acetonitrile, 8–11 min at 80%, 11–12 min from 80 to 10%, then 8 min re-equilibration at 10%. The flow rate was 1 ml/min at room temperature. The eluents were degassed online (Gastorr GT102, FLOM Corporation, Japan).

2.4. LC/MS

LC/MS was performed on an API4000 LC/MS/MS (Applied Biosystems, Rotkreuz, Switzerland) using electrospray in the negative ion mode. Chromatography was performed with a 0.1 M NH_4–acetate buffer at pH 7, using a Lichrospher 100 RP-18, 5 μm 125 mm × 4 mm column (Merck). The following gradient elution with acetonitrile was used: 0–10 min from 0 to 80%, 1 min at 80%, then in 1 min to 0% and re-equilibration for 6 min. Fifty microlitres samples (100 μM FMOC-EDDS) were injected.

2.5. Water and soil solution samples

Tap water samples were taken from the non-chlorinated normal domestic supply. Its calcium content was 2 mM and the magnesium content 0.7 mM.

Soil solution was collected using Rhizon Flex soil moisture samplers (Rhizosphere Research Products, Wageningen, The Netherlands) from two top soils. The first soil was a non-calcareous, acidic, sandy loam with a pH of 5.5 (in 0.01 M $CaCl_2$), the second soil was a non-calcareous, near neutral loam with a pH of 6.6 (in 0.01 M $CaCl_2$). Both soils were agricultural in origin and from Northern Switzerland.

2.6. Plant material extraction

The plant material originated from a hydroponic experiment using sunflowers to investigate the uptake of heavy metals and EDDS from nutrient solution [21] and also a pot experiment investigating the use of EDDS for enhancing phytoremediation. Dried (40 °C) ground plant material from both roots and shoots were extracted in pure water (10 mg/10 ml) by sonication with a micro-tip sonic probe for 1 min. The samples were kept on ice during sonication to prevent heating. They were then centrifuged and filtered (0.45 μm) before derivatization. Xylem sap samples were collected by decapitating the plants and collecting the xylem sap for 2–3 h. The samples were diluted immediately before derivatization by adding 800 μl of pure water (sample weight 4–140 μg).

3. Results and discussion

3.1. Derivatization of EDDS

The derivatization of amino acids by FMOC with borate buffer at pH 8 and at room temperature is complete within 30 s [15,16]. The derivatization of EDDS, however, is much slower. A reaction time of less than 1 min at room temperature is not sufficient for a complete derivatization of EDDS. After heating for 10 min at 60 °C a maximal conversion to the derivative was achieved. However, longer heating times reduced the peak area again. We found that at room temperature maximal derivatization of EDDS with FMOC occurred at a reaction time of 30 min, yielding the same maximal peak area as heating for 10 min at 60 °C (Fig. 1a). The effect of pH on derivatization at room temperature is shown in Fig. 1b. It can be seen that the peak area increased exponentially with increasing pH. An EDTA buffer with pH 11.5 was therefore chosen for the analysis of all natural samples. Some method development was also carried out using an EDTA buffer with pH 8 or a borate buffer with pH 8.

Metals present in the sample may inhibit the derivatization of EDDS. Attempts to remove the cations by passing the

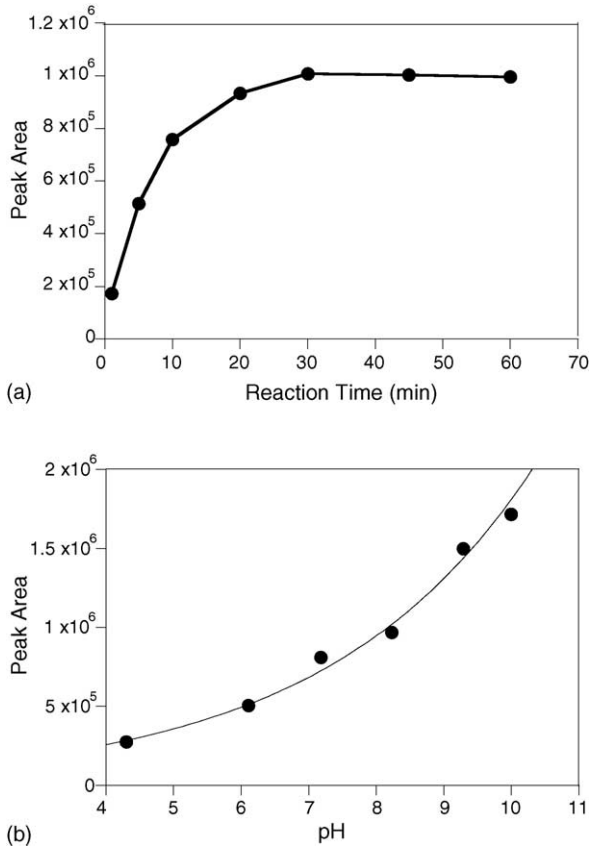


Fig. 1. Influence of reaction time (a) and EDTA-buffer pH (b) on the derivatization of 1 μM EDDS with FMOC at room temperature.

sample through a cation exchange column in the H^+ form were not successful because EDDS was also retained. Addition of the chelating agent EDTA in excess of all metals (10 mM) resulted in a maximal conversion of EDDS to the FMOC derivative in most cases. Concentrations of Ca^{2+} up to 1 mM and of NO_3^- up to 0.1 mM did not affect the derivatization of 1 μM EDDS (Table 1). The addition of 10 μM Zn(II), Cu(II), Pb(II), as nitrates was also investigated but showed no effect. 10 μM Fe(III) reduced the EDDS peak area by 14%, while 10 μM Ni reduced it by 91%. In further tests with concentrations of Fe(III) and Ni up to 100 μM, Ni produced the same reduction whatever its concentration, while Fe(III)'s effect increased with increasing concentrations (Table 1). Heating 1 μM EDDS and 10 μM Ni with EDTA buffer at pH 8 for 3 h at 90 °C before adding the FMOC reagent produced the same signal as 1 μM EDDS in the absence of Ni with and without heating. The same experiments carried out using EDTA buffer at pH 11.5 produced enhanced signals, due to a more efficient derivatization at higher pH. NiEDTA is a complex that is known to react very slowly. Heating greatly increases the reaction rate [22]. As EDDS is an isomer of EDTA, the same can be assumed for NiEDDS. EDDS complexes must yield their metals to EDTA, in order for the free EDDS to be derivatized by the FMOC reagent. Heating accelerates this rate limiting step.

Repeated measurement of a derivatized sample indicated that the derivative was stable for at least 18 days when stored at 4 °C in the dark.

Table 1
Influence of metals on EDDS derivatization by FMOC. Conditions: 1 μM EDDS, addition of EDTA-buffer (pH 8), 30 min reaction with FMOC at room temperature

Conditions	Relative peak area	
	Unheated	Heated[a]
Free	1.00	1.00
1 mM Ca^{2+}	0.96	
100 μM NO_3^-	1.00	
10 μM Cu, Zn, Pb	0.98–1.00	
10 μM Fe	0.86	
10 μM Ni	0.09	0.99
100 μM Fe	0.69	
100 μM Ni	0.09	

[a] Heated for 3 h at 90 °C after the addition of EDTA buffer and before FMOC addition.

3.2. HPLC separation

Fig. 2 shows a chromatogram of 10 μM EDDS in pure water derivatized in EDTA-buffer. The EDDS peak is well separated from the reagent peak (elution time 9 min) and additional peaks originating from impurities in the EDTA (elution time between 7 and 9 min). Some batches of EDTA also gave a small peak originating from impurities, or degradation products emerging over time, that elutes at the same time as EDDS. Careful testing of the purity of the used EDTA batch is therefore necessary in order to ensure that it is free of this interference. A solvent blank sample should also be derivatized with every calibration to check for degradation of the EDTA buffer over time.

For standards made in pure water the relationship between the peak area and the concentration of EDDS was linear from 0.01 to 10 μM (fluorescence detector gain × 10) with a correlation coefficient r^2 of 0.9952 ($n = 10$). The relationship was also linear from 1 to 30 μM (gain ×1) with a correlation coefficient r^2 of 0.9910 ($n = 6$). The detection limit is 0.01 μM ($S/N = 3$).

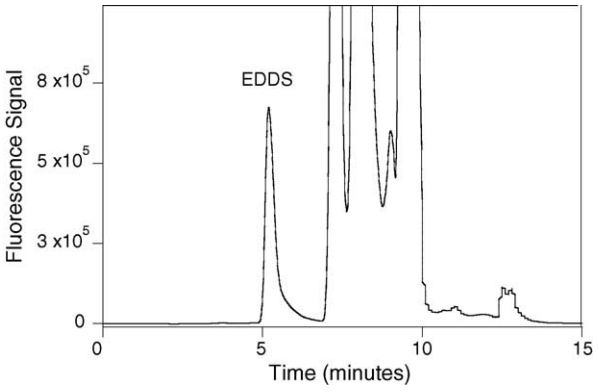


Fig. 2. Chromatogram of 10 μM EDDS in pure water.

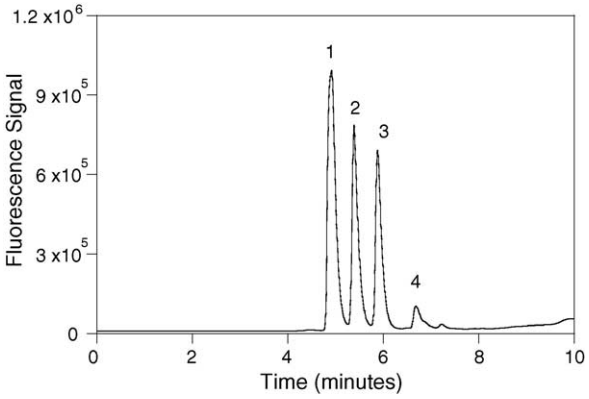


Fig. 3. Chromatogram of 1 μM EDDS derivatized with FMOC in borate buffer at pH 7.7, PLRP-S polymer RP-C18 column (see Section 3.3 for details).

3.3. Identification by LC/MS

Fig. 3 shows a chromatogram for EDDS derivatized in borate buffer without addition of EDTA (PLRP-S polymer RP-C18 column). Borate instead of EDTA buffer was used in LC/MS analysis because the derivatization in EDTA buffer gives additional overlapping peaks which obscure the EDDS signals (peaks 2–4) and thus complicate structure assignment.

The EDDS standard used yielded four distinct peaks. The first peak consists of a compound with a molecular ion of *m*/*z* 513 which corresponds to a singly derivatized FMOC-EDDS (see Scheme 1). MS/MS of this ion gave fragments at *m*/*z* 495 (–H_2O), 397 (loss of maleic acid), 317 (loss of fluorenyl-methanol), 291 (EDDS; loss of FMOC), 273 (291 minus water and cyclization), 229 (elimination of CO_2 from 273, one of three possible structures is shown), 157 (loss of maleic acid from 273).

The *m*/*z* of the molecular ion of peak 2 was 495, indicating that EDDS had undergone cyclization before or during derivatization, as described by Kolleganov et al. [23]. Elimination of FMOC from this ion yields *m*/*z* 273, followed by loss of CO_2 (one of three possible structures is shown) or maleic acid (see Scheme 2).

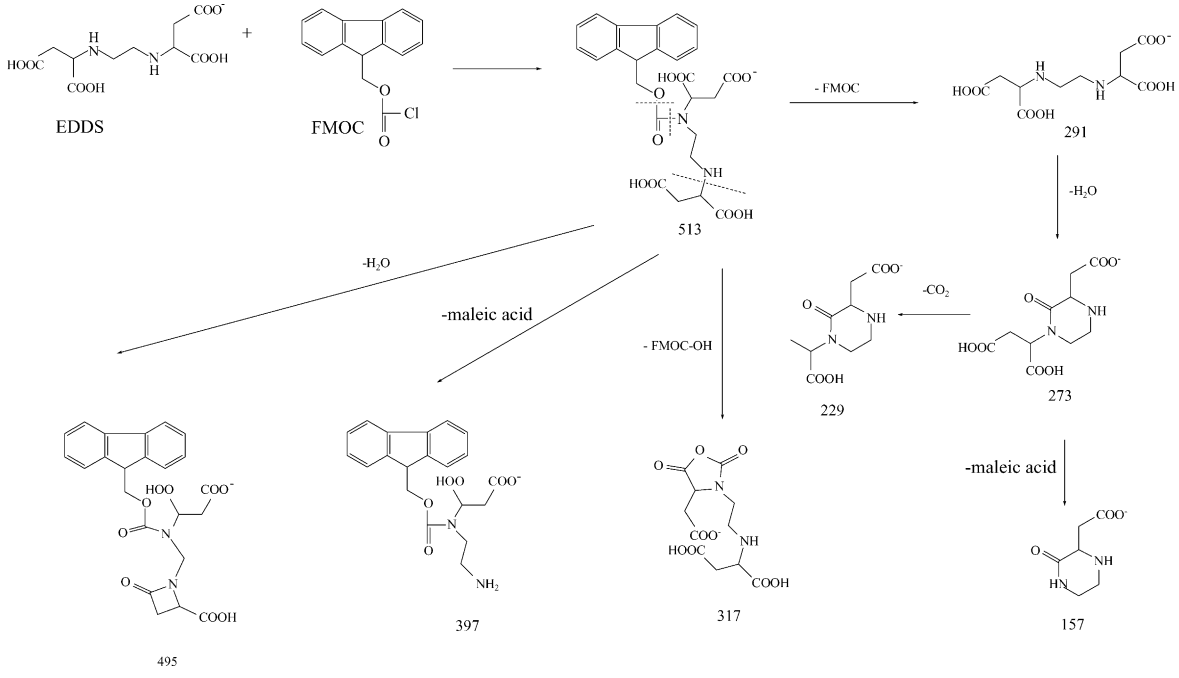
Peak 3 corresponds to a deprotonated molecular ion with a *m*/*z* of 354. Its fragmentation pattern indicates FMOC-aspartate. The *S*,*S*′-EDDS used had been synthesized from L-aspartic acid and 1,2-dibromoethane. This peak thus was likely an impurity in the EDDS standard. The major masses were *m*/*z* 158 (elimination of fluorenyl-methanol) and 165 (formation of the fluorenyl anion). Scheme 3 shows the proposed fragmentation pattern.

Peak 4 had a molecular ion of *m*/*z* 735, which corresponds to the derivatization of both imine groups of EDDS. The fragmentation yielded *m*/*z* 513 (elimination of one FMOC) and further all the masses observed in peak 1 (Scheme 1).

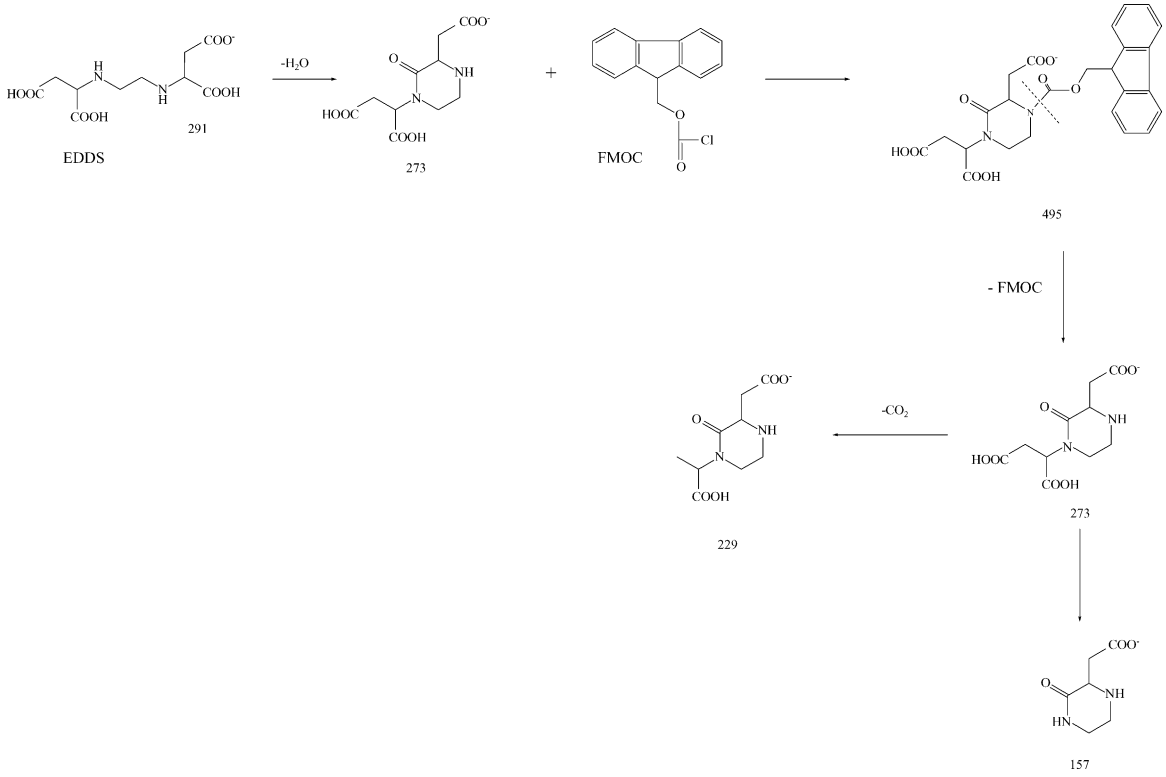
Based on the data presented, the first peak observed in the chromatogram was used for quantifying EDDS, since it clearly corresponds to the derivatized EDDS, while the other peaks could be assigned to reaction side-products.

3.4. Analyses

Both tap water and two types of soil solution were spiked with EDDS over a concentration range of 0.01–10 μM and the results compared to pure water standards. Tap water was found to reduce the peak area by 5%. The ten times diluted



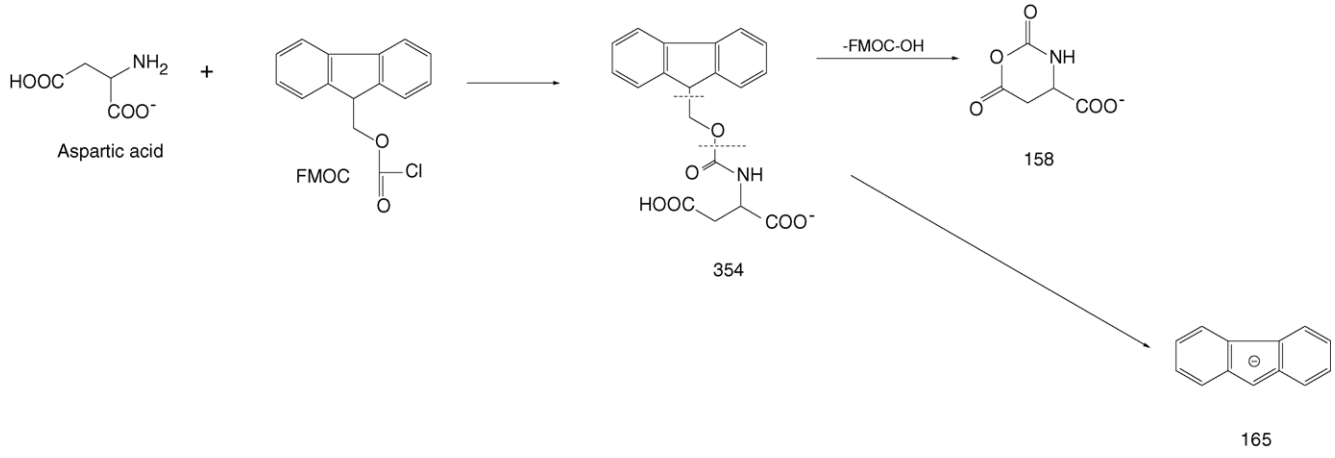
Scheme 1. MS/MS fragmentation pattern of the negatively charged molecular ion *m*/*z* 513 of peak 1 in Fig. 3.



Scheme 2. Fragmentation pattern of the molecular ion *m*/*z* 495 of peak 2 in Fig. 3.

soil solutions both gave a 7% reduction in peak area and the undiluted soil solutions a 9–10% reduction. For soil solution or other natural water samples we have therefore always prepared the standards in the same EDDS-free matrix as the samples. Where an EDDS-free matrix is not available, standard addition has to be used for the samples. In addition real soil solution samples from a soil washing experiment with EDDS were successfully quantified (undiluted and diluted 10 and 50 times). Fig. 4 shows an undiluted soil solution sample containing 0.75 μM of EDDS.

There is only one other detailed report on the analysis of EDDS in natural waters [14]. This method is based on the ion chromatographic separation of Fe(III)EDDS and UV-detection. In distilled water the detection limits of both methods are the same. The Fe(III)EDDS method, however, suffers from matrix effects by major ions (e.g. chloride, sulfate, phosphate). The observed peak broadening and peak area reduction results in a reduced sensitivity in natural waters which limits the applicability of the method to well defined matrices.



Scheme 3. Fragmentation pattern of molecular ion *m*/*z* 354 of peak 3 in Fig. 3.

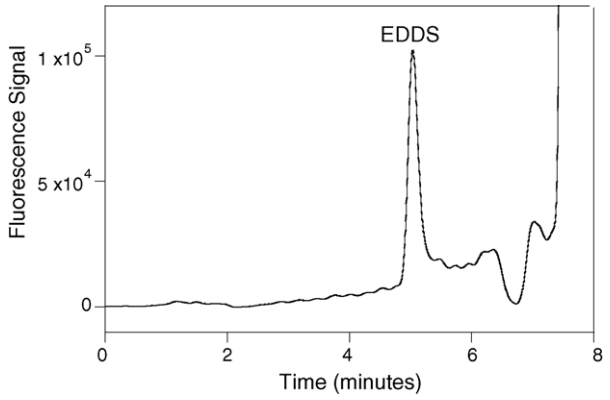


Fig. 4. Chromatogram of an undiluted soil solution sample containing 0.75 μM EDDS.

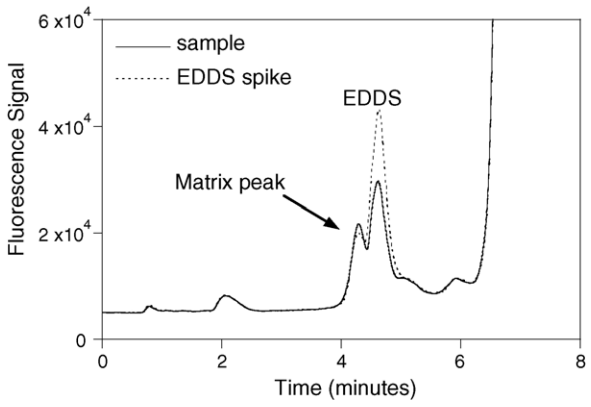


Fig. 6. Chromatogram of plant xylem sap with spike (0.5 μM EDDS).

Using plants grown in the presence of EDDS (see Section 2.6) it was found that extracts of shoots and roots could be successfully analysed after FMOC-derivatization (Fig. 5). Sub-samples were also spiked with EDDS prior to derivatization in order to help identify the EDDS peak among the plant matrix peaks at low concentrations. Fig. 5a shows a chromatogram of a shoot extract with an EDDS concentration of 0.19 μM from the hydroponics experiment and also the sample spiked with 0.2 μM EDDS which gave a recovery of 91%. The actual shoot concentration was 183 μmol/kg. Fig. 5b shows a root extract from a pot experiment where much higher concentrations of EDDS were found. The extract concentration was 6.43 μM EDDS and the spike concentration was 2.5 μM with a recovery of 97%. The actual root concentration was 4791 μmol/kg.

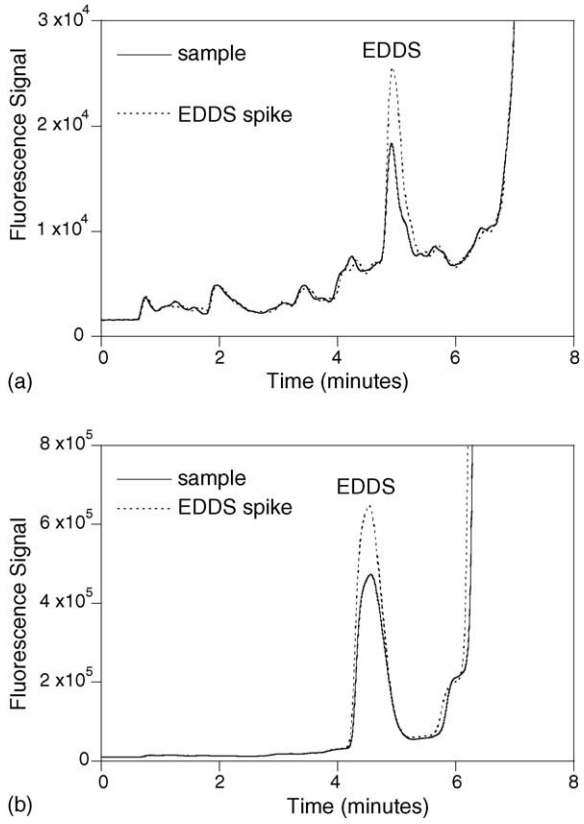


Fig. 5. Chromatogram of (a) an extract of a low concentration shoot sample (0.19 μM EDDS) spiked with 0.2 μM EDDS and (b) an extract of a high concentration root sample (6.43 μM EDDS) with spike (2.5 μM EDDS).

Plant xylem sap samples were also analysed. Some interference from the matrix, which produced a partially co-eluting peak with the EDDS peak, could not be overcome by changing the gradient. The spike recovery was between 30 and 90% for these samples. Small sample volumes (few μl) in some cases may have led to inaccuracies due to the large dilution factors required to be able to analyse the samples. Fig. 6 shows a sample and corresponding spiked sample with a concentration of about 0.23 μM EDDS and a spike of 0.5 μM EDDS. In this case the spike recovery was 47%.

Xylem sap and plant material analysis was not possible using the Fe(III)EDDS method [14] due to co-eluting compounds and a large reduction in peak area and excessive peak broadening.

4. Conclusions

The results show that the chelating agent EDDS can be derivatized using FMOC to give derivatives suitable for separation by reversed-phase HPLC. The method is applicable to the determination of the compound in water, soil solution and plant material at trace levels.

Acknowledgements

We thank René Schönenberger for the help with the HPLC-MS measurements and Diederik Schowanek from Procter & Gamble for providing *S,S*-EDDS. This work was funded in part by the Federal Office for Education and Science within COST Action 837 and the Swiss National Science

Foundation in the framework of the Swiss Priority Program Environment.

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