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DEVELOPMENT AND IMPROVEMENT OF DETECTION AND CONFIRMATION PROCEDURE IN THE ANALYSIS OF MARINE TOXINS: DETERMINATION OF OKADAIC ACID IN MUSSELS

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DETERMINATION OF OKADAIC ACID IN MUSSELS

CONTENTS

ABSTRACT

The okadaic acid (OA) is one of the major toxins responsable of diarrhetic shellfish poisoning (DSP), which are produced by marine dinoflagellates.

The quantitative determination of OA in shellfish samples has been investigated on basis of the method developed by Lee et al. (1987). This method includes consecutive extractions with methanol and chloroform suitable for this rather lipophilic carboxylic acid, and a fluorescence labeling with 9-anthryldi-azomethane (ADAM), a derivatisation reagent highly specific for carboxylic acids.

Derivatisation of OA with ADAM synthesized in situ was tested and proved to be satisfactory reproducible. The derivatives were stable. However, as the derivatisation reaction was not complete with this ADAM synthesis mixture, a large excess of ADAM is required to assure the labeling of all the present acid.
The deoxycholic acid (DCA), was tested for derivatisation with ADAM, and used as an internal standard for the OA determination in order to avoid errors due to uncomplete derivatisation of OA. The stability of the DCA was established , allowing the control of OA standard in solution.

Conditions of extraction have been examined. The pH and a reverse phase chromatography of the methanolic extract had an influence on both derivatisation yield and extraction recovery. In conclusion, it is suggested to control the pH of extraction (to be set at 7.1 to 8), and to apply clean-up of the extract before derivatisation.

1 INTRODUCTION

The okadaic acid (OA or 9,10 deepithio-9,10-didehydroacan-thifolicin), is named from the sponge it was first isolated. This component is a C 38 polyether fatty acid, and is one of the toxins responsable of diarrhetic shellfish poisoning (DSP) in Europe, causing severe gastrointestinal illness. When occurring, OA is the major toxin in the seafood in Europe.

Okadaic acid forms a white cristalline solid with a melting point of 156-158°C, which is soluble in ether, acetone and ethyl acetate, but slightly soluble in chloroform and methanol. The compound has no absorption maximum in UV region, which will impose a method of derivatisation for chemical detection.

The OA is a polyether of ionophore type, similar to the mono-carboxylic polyether antibiotics like Monensin. For chemical determination, the procedure for OA will be very similar to those of these antibiotics. OA can be extracted under ion pair form : a partition experiment showed that 80% of OA is distributed in the organic phase when equilibrated between chloroform and a buffer at pH 7.1 (20 mM phosphate)(Allenmark et al., 1990).

Unlike the PSP toxins OA has rather hydrophobic properties and therefore the analysis of samples will be different and also more complicated.

Since the isolation of OA by Tachibana (1981), this toxin has been determined in sea products by several methods. First the bioassays were developed, such as LD 50 (Yasumoto, 1980) or diarrhetics tests in mice and rats (Kat, 1983; Edebo et al.,-1989). These methods however allow only global determination of all DSP toxins and are not specific for OA. Later, physical and chemical techniques have been developed as well as immunologi-cal techniques (Uda et al., 1989).

But for all these techniques, the major problem remains the availability of a pure OA standard.

Isobe et al. (1986) have developed a method for synthesis of OA, from D-glucose derivatives and butyne-diol and consisting of 106 steps. Beside this time consuming synthesis proces, the OA can be isolated from sufficiently contaminated shellfish or from dinoflagellates cells (Murakami et al., 1982; Yasumoto et al., 1985; Kumagai et al.,1986).

This procedure comprises generally consecutive extractions with acetone and diethyl ether, several clean-ups by column chro-matography (basic alumina), gel permeation chromatography and several purifications on reverse phase liquid chromatography. Different monitoring and confirmation techniques for

OA were used to check the extracts during the purification, such as: the mouse bioassay, TLC on silicagel with detection by heating after 50% H2SO4 spraying and GLC of extract performed after trimethylsilysation.

For quantitative determination of OA in mussels or other shellfish, the method should be more sensitive than these techniques in order to detect concentrations of µg/g matrix. This means quantitative extraction procedures and highly sensitive detection.

Lee et al. (1987) have developed a method by HPLC, including a derivatisation procedure with 9-anthryldiazomethane.

The extraction of OA is not carried out on the whole mussel, since OA has been found to be localised in the hepatopancreas of the shellfish (Yasumoto, 1980). Edebo et al. (1988) report for contamined mussels, that OA concentration in gills, mantles and adductor muscles and remainder amounted to less than 2% of that in the hepatopancreas.

The recovery will depend on the way to extract the acid from the biological matrix. Edebo et al. (1988) showed the importance of the disintegration of the matrix. Comparing different techniques of homogenisation of the fresh and not cooked hepatopancreas, they found that the recovery was optimal if the matrix was finely dispersed and subsequent vigourous extracted with methanol.

In general, the analysis remains very delicate because OA is easily broken down by daylight, and seems sensitive to oxidant trace in solvent. However, tested with boiling water the stability of OA is good (Edebo, 1988), in the hepatopancreas after homogenisation. Only after prolonged boiling the concentration of the OA was significatively reduced (in order to reduce the concentration with 50%, boiling for ca 3h was required).

The method of Lee was adapted using combined liquid chromatography and mass spectrometry and ion-spray ionisation (Pleasance et al., 1990).

Given the nature of OA to be in a ionic form, easily extractible from water solution, derivatisation can be used where the carboxilic acid can act as a nucleophilic (anion), to a activated halogen component. So OA has been derivatised with N-(9-acridinyl)-bromoacetamide that offers an enhanced fluorescence intensity. But this reagent has a low selectivity because it reacts also with the amino or thiol compounds that can be present in the matrix extract (Allenmark, 1990).

In this report the method for the okadaic acid determination has been investigated on basis of the method of Lee et al. (1987). We have investigated the derivatisation with the same reagent, prepared with an in situ method, that presents some advantages.

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The use of an internal standard was developed and the conditions of extraction were examined.

2 DERIVATISATION WITH 9-ANTHRYLDIAZOMETHANE SYNTHESISED IN SITU

2.1 INTRODUCTION

The 9-anthryldiazomethane (ADAM) has been widely used as derivatisation reagent for the HPLC determination of several types of carboxylic acids, first for fatty acids (Nimura & Kinoshita, 1980), fatty acids in serum (Ichinose et al, 1983; Ghiggeri et al, 1986; Shimomura et al, 1986; Hatsumi et al., 1986), for prostaglandins (Hatsumi et al, 1982), further for oxalic acid in urine (Imaoka et al, 1983), amino acids (Yoshida et al, 1985), and Monensin and other ionophore antibiotics of the same group (Takatsuki et al, 1986; Martinez and Shimoda, 1985 and 1986).

ADAM allows a highly sensitive detection thanks to the strong fluorescence of the anthracene group. Moreover, the use of ADAM offers the remarkable advantage that, in a matrix containing amino acids, it will not alkylate an amino group, under mild conditions (Yoshida et al, 1985).

First synthesis of ADAM was carried out by Nakaya et al. (1967), using mercury-1-oxide as a catalyst for the oxidation of 9-anthraldehyde hydrazone. Barker et al. (1980) then proposed a quicker procedure using activated manganese dioxide. In both preparation procedures, the ADAM must be purified since some of the inorganic oxidant remains in the reaction mixture even if the reaction is completed. If the reaction mixture was directely used for derivatisation, the carboxylic acids susceptible of oxidation could be destroyed. However, after purification, the stability of ADAM during storage is not sufficient. In literature, the stability of ADAM varies ; as a crystal, ADAM can be stored at -20°C at least one month; in ether solution, ADAM must be used within 5 months when stored at -20°C, or within 3 weeks at 4°C.

For these reasons, the preparation in situ proposed by Yoshida et al, (1988) was tested for the OA determination . This synthesis procedure uses the strong oxidising agent N-chlorosuccinimide to oxidise the substrate 9-anthraldehyde hydrazone, with quinuclidine as a catalyst.

The synthesis in situ offers the advantage that the ADAM reagent does not need to be purified. The shortcoming caused by degradation of ADAM is overcome in this way, since for each new derivatisation, a new ADAM is synthesised in situ and used immediately.

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However for the application of this procedure several conditions must be fulfilled and verified: the reagents still present in the synthesis mixture should not interfere in the derivatisation process with acids and should allow good detection and separation of the ADAM derivatives in the HPLC system.

In order to test this ADAM synthesised in situ, we used a bile acid, the deoxycholic acid. Easily available and stable, it offers the advantage of having a rather similar polarity as OA (figure 2.0).

Figure 2.0 : Chemical structures
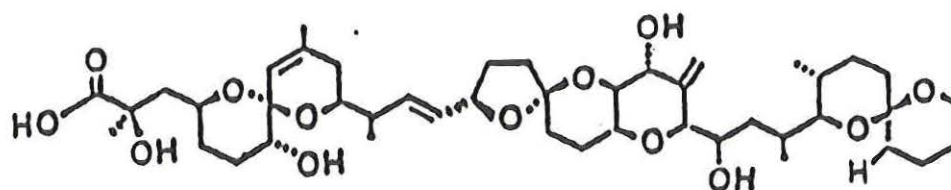
Okadaic acid (OA)

Deoxycholic acid (DCA)

9-Anthraldehyde hydrazone

9-Anthryldiazomethane (ADAM)

ADAM + RCOOH ⟶
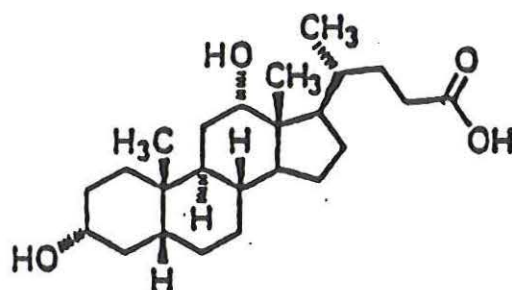
Preparation of 9-anthryldiazomethane(ADAM) according to Yoshida et al (1988), and derivatisation reaction with an acid.

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2.2 METHOD

As the ADAM synthesis mixture and ADAM derivatives are unstable when exposed to light at room temperature, all preparative operations have been done in a room shielded from day light, with subdued lighting.

2.2.1 **Reagents**

- 9-anthraldehyde, 97% Aldrich # 27, 868-8
- hydrazine hydrate, Aldrich # 22, 581-9
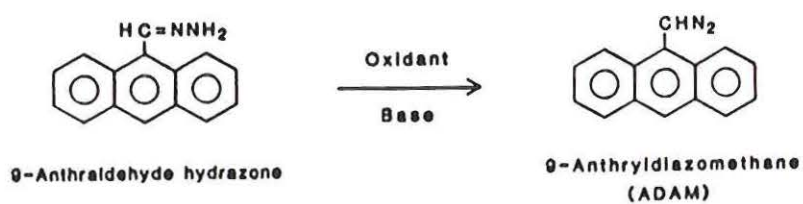- N-chlorosuccinimide 98%, Aldrich # 10, 968-1.
- quiniclidine 97%, Aldrich # 19, 760-2
- deoxycholic acid (DCA) reference standard, Aldrich # 10,   730-730-1
  - stock solution: 10 μg/ml in methanol, stored at 4°C
  - working solutions: from 10 to 0.5 μg/ml by pipetting and dilution of stock solution in a 4 ml glass-vial.
- solvents (Merck): chloroform, ethanol, hexane, methanol were analytical grade; ethyl acetate was distilled; acetonitrile was chromatography grade. All solvent mixture were freshly prepared before each use.

2.2.2 **Apparatus and operating conditions**

- Heating module, Reacti-Therm, Pierce :
  - metallic thermostatic block for test tubes, with nitrogen flowing into each tube.
- HPLC system:

    Column : two cartridges of 100x30 mm id. glass columns packed with RP Chromspher C-18, 5 μm particle size (Chrompack # 28267) with a 10 mm guard column.

    Injection: Water Intelligent Sample Processor (WISP), 710B automatic, injection of 10 μl from the methanolic solutions.

    Pump: Waters M45
    operating conditions: 0.5 ml/min of acetonitrile/water 90/10 (v/v).

    Detection: fluorescence detector Hitachi-Merck F-1020 operating conditions: excitation wavelength at 365 nm, emission at 410 nm. Sensitivity at 1.0.

    Recorder: Kipp & Zonen BD40 with double marker pens operating conditions: paper speed: 5 mm/min, sensitivity at 10/20 mV.

- FTIR analysis:

    Bruker IFS-85 (Bruker Analytische Messtechnik GmbH, 7500 Karlsruhe 21, Germany)
    Source: Globar
    Detector: DTG S, cuvette 0,02 cm with potassium bromide windows
    Resolution: 2,0; number of scans: 200.

2.2.3 Synthesis of 9-anthraldehyde hydrazone according to Nakaya et al. (1967)

Crystals of 9-anthraldehyde, 8.8 g in total were dissolved in 150 ml absolute ethanol, in a 250 ml erlenmeyer. The hydrazine hydrate (9 ml) was added and the solution was stirred for three hours at room temperature. The solid product was filtered off under low pressure (Whatman nº 5 filter) and dried under vacuum. The solid was purified by recristallisation from ethanol, filtered off and washed with the mother solution and finally dried. The crystals have a melting point of 124-126°C (Nakaya et al., 1967).

The light yellow crystals were stored at -18°C. The solution in ethyl acetate was stored in the dark, and used within two months.

2.2.4 Preparation of ADAM synthesis mixture according to Yoshida et al. (1988).

For each derivatisation, a new batch of ADAM synthesis mixture was prepared. In a 4 ml-glass vial (WISP vial) with screwed cap, 500 μl of the following solutions in ethyl acetate were mixed: N-chlorosuccinimide 6.9 mM, 9-anthraldehyde hydrazone 6.9 mM and quinuclidine 69 mM. The resulting mixture was allowed to stand at room temperature in the dark. After 30 min of reaction time, this solution was used for derivatisation.

The ethyl acetate solutions of these three components were stored in the dark, at room temperature, and used within two months.

2.2.5 Derivatisation with ADAM

The derivatisation with ADAM was carried out in a glass micro-tube (300 μl vial insert for WISP vials). 100μl of the ADAM synthesis mixture was added to 100μl or 200μl of the deoxycholic acid solution. The solution was Vortex-mixed. The vial was placed in a heating-block and was allowed to stand one night at 50°C.

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2.3 RESULTS AND DISCUSSION

2.3.1 Parameters of derivatisation

Under the chromatographic conditions described above the DCA derivative, eluted at retention time of 14 min. Peaks eluting earlier were ADAM, decomposed products of ADAM and impurities. Factors influencing the derivatisation process have been studied in order to optimise the fluorescence response on HPLC. The excitation and emission wavelengths, respectively 365 and 410 nm, were selected as described in literature (Yoshida et al., 1988).

First the kinetics of DCA binding to ADAM was examined. The esterification of the acid was accelerated at higher temperature (Fig 2.1). With an incubation at 50°C, the binding of DCA to ADAM was time-dependent; after 6 hours of incubation the reaction reached a plateau. No degradation of the derivatives was observed after standing 24 h at 50°C. Therefore the overnight derivatisation was applied in subsequent experiments.

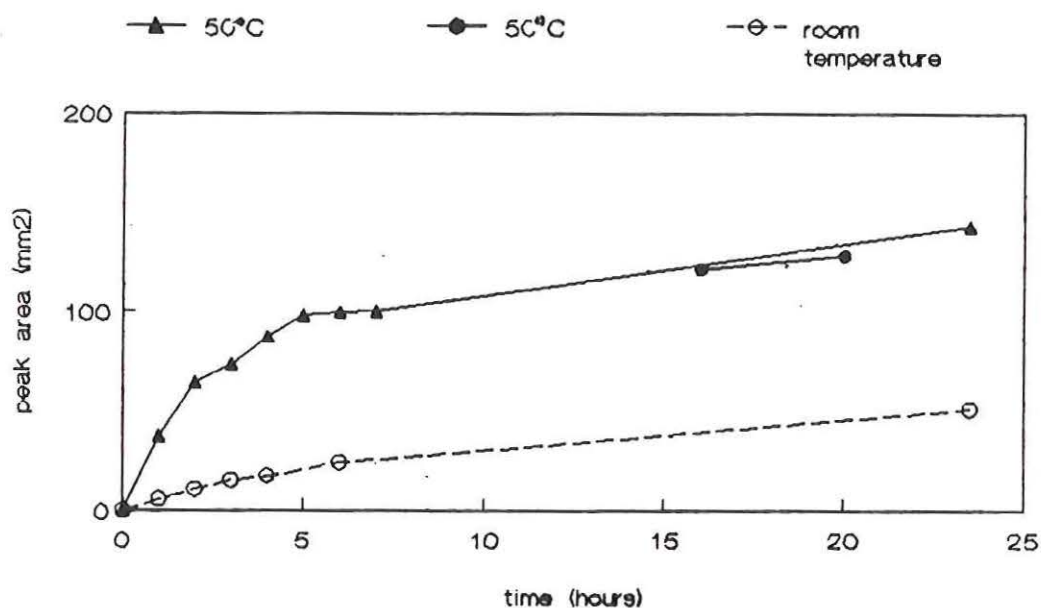Figure 2.1 : Time dependence of DCA reaction with ADAM, at room temperature and at 50°C. Derivatisation of 1 μg DCA in a total volume of 300 μl (each line corresponds to one derivatisation vial from which at several times 10 μl samples where taken out for analysis).

At this temperature the volume of the reaction mixture could become inaccurate because of solvent evaporation. However the good reproducibility of the derivatisation procedure (see further) showed that this factor was not significant.

The rate of reaction of ADAM with deoxycholic acid was fairly low. As reported in literature, the rate of reaction with pure ADAM solutions depends on the polarity of the solvent. Longer reaction times are reported in a low-polar solvent like ethyl acetate (Imaoka et al., 1983) and in methanol/diethyl ether (28/72) (Takatsuki et al., 1986). To derivatise fatty acids with pure ADAM solutions in ethyl acetate, an incubation at 40°C for 30 min was used by Hatsumi et al (1982 and 1986).

In order to investigate the effect of the ethyl acetate proportion on the derivatisation response, derivatisations of constant amount of DCA were carried out in a total volume of 300 μl, in methanol/ethyl acetate 1/2 and 2/1. With a reaction time of one night, no significative differences were observed in the derivatisation yield.

2.3.2 The synthesis of ADAM in situ

When 9-anthraldehyde hydrazone is mixed with the oxidising agent and the catalyst, the color of the solution turned from a pale yellow to light orange after 30 min. ADAM itself has a red orange color as crystal (Nakaya et al, 1967).

In order to check the influence of the relative concentration of the three reagents on the synthesis of ADAM, the concentrations of the solutions were varied and the synthesis verified with derivatisation of DCA. With a twice as high concentrated of N-chlorosuccinimide, the response of DCA was much lower, and the synthesis mixture of ADAM had a light yellow color, indicating that the synthesis was worse, and that ADAM was rapidly oxidised. The double quantity of catalyst quinuclidine gave no higher response of DCA derivative.

Other solvents for the preparative solutions were tested. As mentioned in Yoshida et al.(1988), acetone gave a lower ADAM yield due to the reaction of hydrazone with acetone. In diethyl ether, the N-chlorosuccinimide was not readily soluble. The ethyl acetate was then kept as solvent, despite the fact that this solvent contains traces of acetic acid; this acid was reported (Takatsuki et al., 1986) to react quite sponteanously with ADAM. This gave derivatisation by-product in the chromatogram, and limited the time of use of the synthesis mixture.

Due to the fact that ADAM must be in excess present for derivatisation, it was necessary to check the progress of the synthesis, and to determine the concentration of ADAM synthesised in situ. Yoshida et al.(1988) checked the stability of ADAM in the synthesis mixture, but no synthesis yield nor concentration of ADAM in the final mixture was determined. Two different ways were available to establish a quantitative or a relative concentration of ADAM: the first is FT-IR measurement, the second is based on the determination of derivatisation yield with DCA.

2.3.2.1 FT-IR analysis

The FT-IR analysis have been performed on the ADAM synthesis mixture. The absorbance band at 2040-2090 cm-1 is characteristic for the diazo group (-N≡N) and allows identification of ADAM (Nakaya et al., 1967) as the solvent ethyl acetate and the three preparative solutions of quinuclidine, N-chlorosuccinimide and the 9-anthraldehyde hydrazone do not show absorption in this region. It can be seen that no absorption peaks were present in the region of 2060-2080 cm-1. For the last compound the whole spectrum was determined (Fig 2.2). The high absorption peak at 730 cm-1, caused by the out-of-plane vibration of C-H bindings on the benzene ring. This peak allows to check for the presence of aromatic hydrocarbons for both 9-anthraldehyde hydrazone and ADAM.

For each analysis of the synthesis mixture, only two spectrum windows were measured, respectively at 2090-2040 cm-1, like presented in Fig 2.3, and 760-710 cm-1. The absorption band of the diazo group was present at 2063 cm-1.
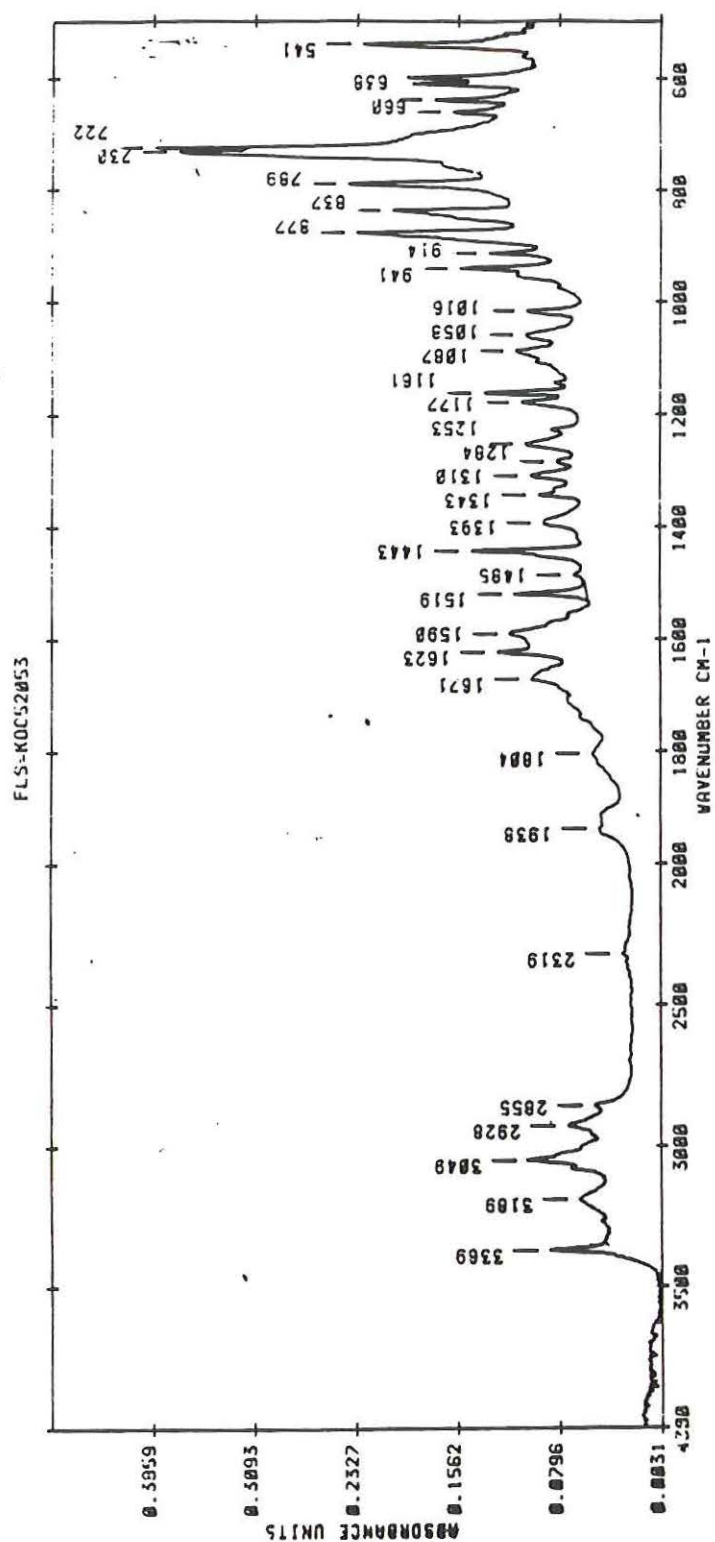
11

Figure 2.2 : FT-IR spectrum of 9-anthraldehyde hydrazone
2.3mM in ethyl acetate.

FLS-KOC42046
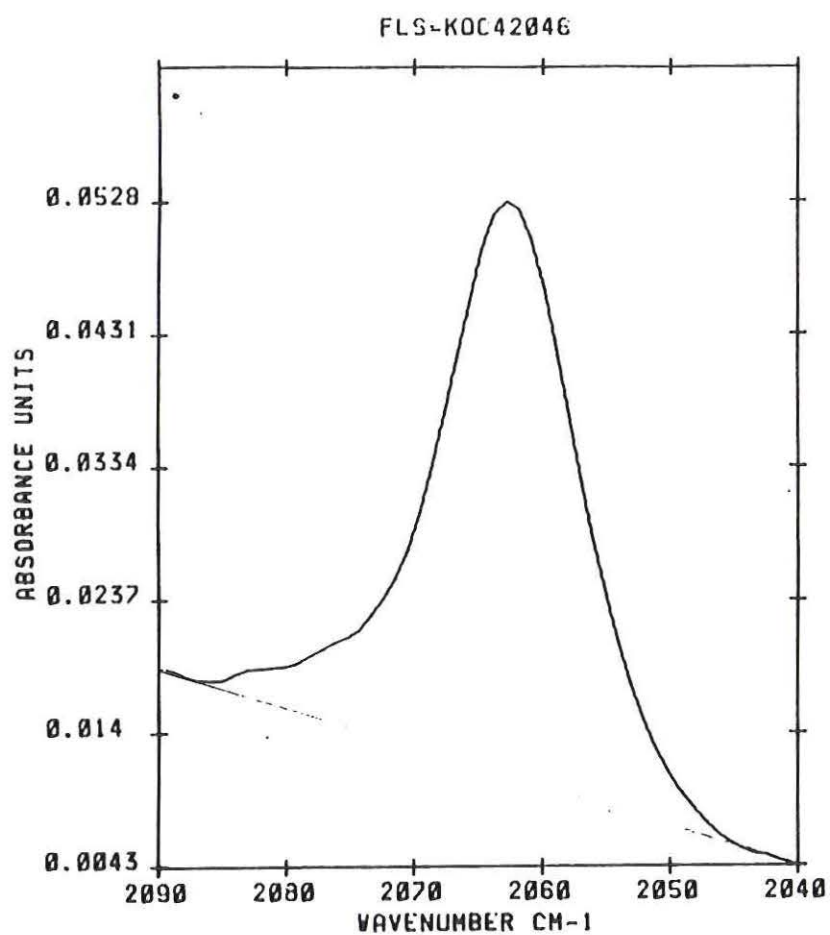
Figure 2.3 : Absorbance band of ADAM in synthesis
mixture.

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When the mixture was measured with continueous IR exposition, a rapid degradation of the synthesised ADAM was observed (Fig 2.4).
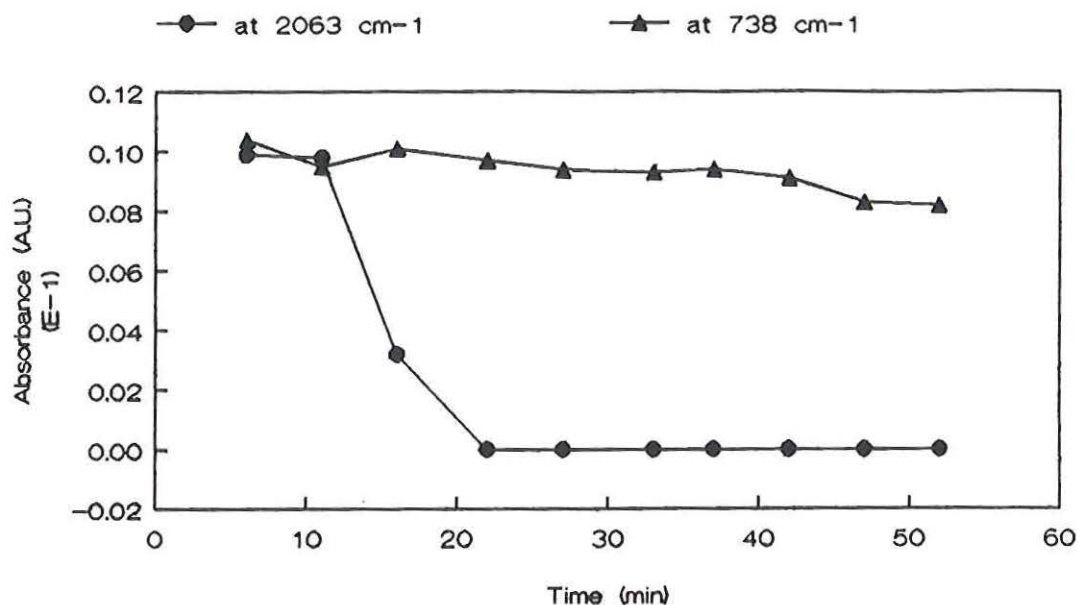
Figure 2.4 : Absorbance at 2063 cm-1 and 730 cm-1 of the synthesis mixture, when measured contineously.

Therefore the IR measurement time was limited to 5 min and the solution was left in the cuvette. The diazo specific absorbance of two different synthesis mixture has been measured after various reaction times in the dark (table 2.1). No significant difference in absorbance was observed, indicating the good stability of ADAM during 17,5 hours. The absorbance band didn't vary after one night standing in the dark at room temperature, but disappeared after one night of incubation at 50°C.

TIME		ABSORBANCE (AU)	
		exp 1	exp 2
	26 min	0.0391	-
	39 min	0.0436	-
	42 min	-	0.0409
1h	38 min	-	0.0373
17h	30 min(18°C)	0.0405	
17h	52 min(50°C)	0.0039	

Tab 2.1 : Stability of ADAM in the synthesis mixture :
Absorbance at 2063 cm-1 at various times; volumes of
9-anthraldehyde hydrazone, N-chlorosuccinimide and quinucli-
dine solution were 1 ml in experiment 1, and 0.5 ml in experi-
ment 2.

The quantitative determination of ADAM in the synthesis mixtu-
re was possible with the molar absorption coefficient deter-
mined at RIKILT-DLO (Tuinstra et al, 1991). This coefficient
was determined in diethyl ether solutions of pure ADAM between
2.31 and 46 mmol/L. The coefficient was 809 L/mole*cm (VC
9.5%).

From the value of absorbance in table 2.1, the concentration
can be calculated by the Lambert-Beers law:

A= C*ϵ*d,

where d is the cell length (0.02 cm), ϵ is the molar absorpti-
on coefficient (L/mole*cm), and C the concentration of the
analyte (mole/L). This gave a concentration of 2.49
± 0.17 mmole ADAM/L (n=4) in the synthesis mixture, resulting
in a synthesis yield of about 90%. (In these experiments the
9-anthryl hydrazone concentration was 2,69 mM/L.

2.3.2.2 HPLC analysis

The principle of the HPLC determination (Tuinstra et al.,
1991) is that ADAM irreversible reacts with an acid to relea-
se nitrogen. If increasing concentrations of DCA are derivati-
sed with a constant and excessive amount of ADAM, the fluores-
cent response will linearely increase untill the acid ADAM is
not in excess any more. The response will then be constant
above a level of DCA called the "overdose". In the linear part
of the plot obtained with an excess of ADAM, the DCA is thus
totally derivatised.

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Derivatisation of various but unknown amounts of ADAM will
therefore be carried out with a overdose of DCA. Because the
DCA is in excess, all the ADAM is supposed to react, and the
fluorescent response will be proportional to the ADAM quanti-
ty. This response can be directly interpreted in mole of DCA
with the help of the calibration curve obtained with excess of
ADAM. Supposed that one mole of DCA reacts with one mole of
ADAM, the calculated quantity of DCA in mole corresponds to
the same quantity of ADAM.

Increasing amounts of DCA were derivatised with 100 μl of the
unknown ADAM solution. The responses from the DCA derivatives
were plotted against the quantity of DCA in the mixture (Fig
2.5). Peak areas of DCA derivatives are first linearly increa-
sing. At 63 nmole derivatised DCA, the response levels off,
meaning that, from that level, not enough ADAM was present.
When DCA was at a level of 509 nmole (200 μg), derivatised
with 100μl of ADAM synthesis mixture, the response of the
derivatives didn't increase at all. This overdose concentrati-
on of DCA was then used for reaction with various amounts of
ADAM (Fig 2.6). With the calibration curve of Fig 2.5, the
response was converted in the amount of ADAM that reacted; the
amount of ADAM was 95.7 ± 2.8 nmole (n=5) in 100 μl used for
derivatisation, meaning that the synthesis mixture had a
concentration of 0.95 ± 0.03 mmole/L, resulting in a synthesis
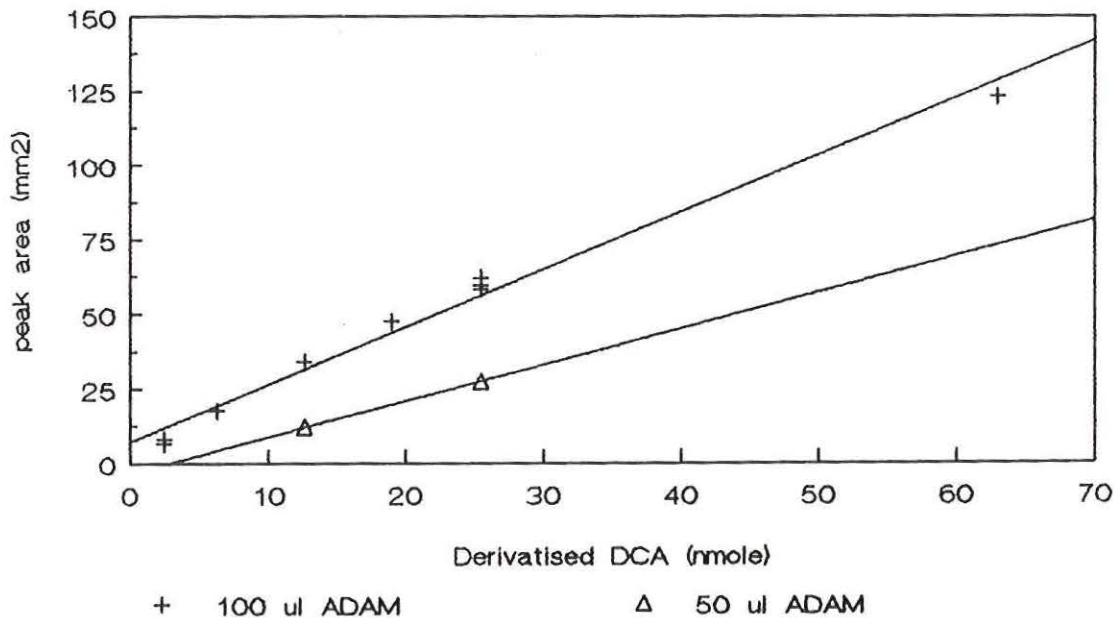yield of only 40%.
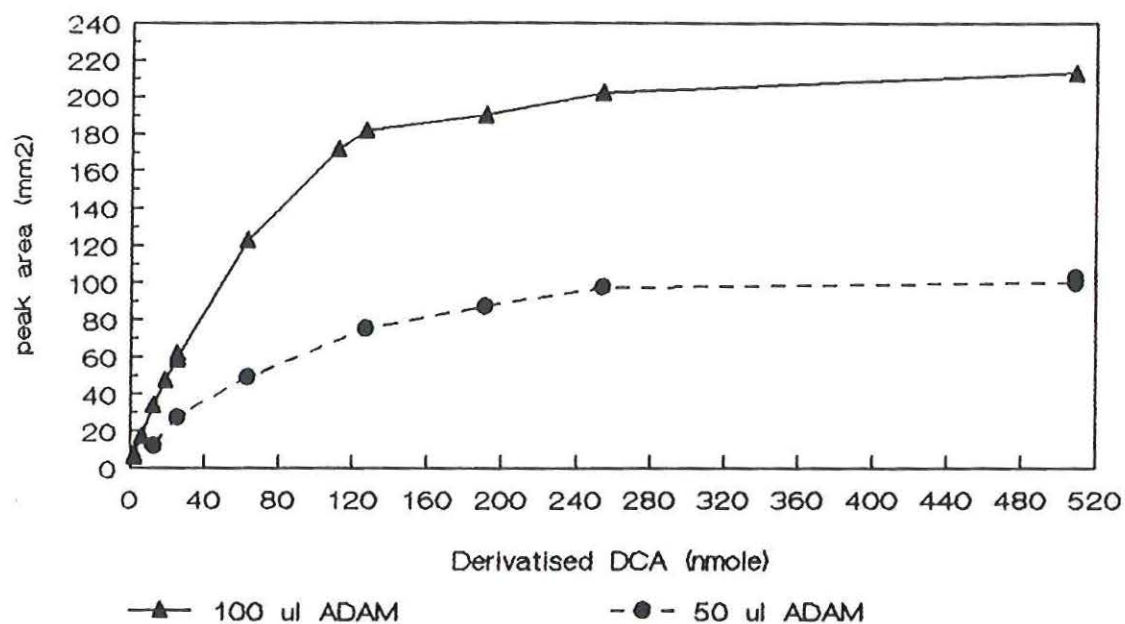
linear part

Figure 2.5 : Derivatisation of increasing DCA concentra-
tion with excess of ADAM; 100 μl and 50 μl of ADAM solu-
tion in a total volume of 300 μl.

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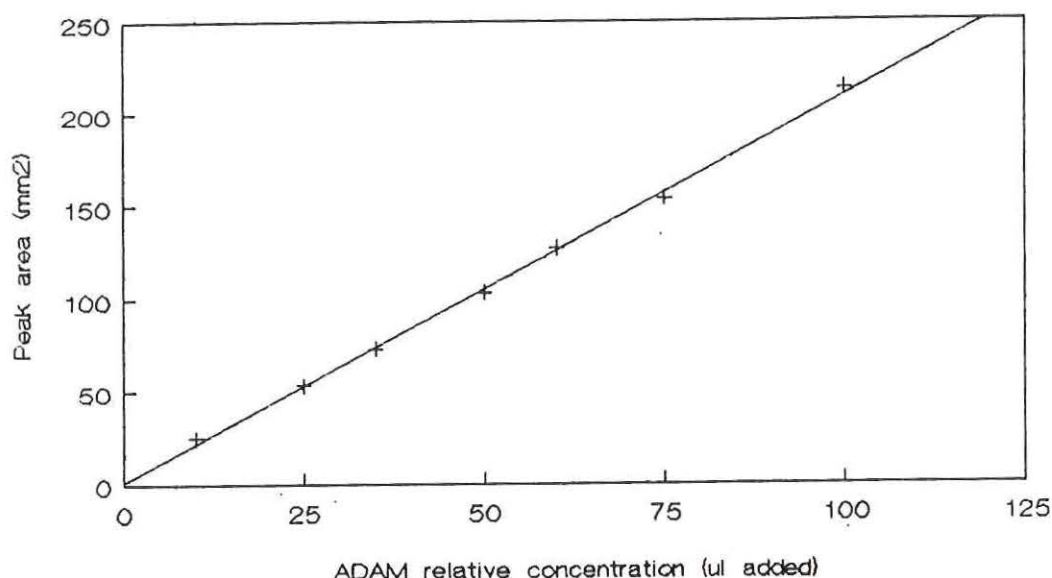
Figure 2.6 : Derivatisation of DCA in overdose with
various volumina of ADAM synthesis mixture; 200 μg or 509
nmole DCA were derivatised in total volume of 300 μL.

2.3.2.3 Conclusion

The synthesis yield calculated from the derivatisation of DCA
reached only 40%, and doesn't agree with the result of FT-IR
which gave 90% of synthesis yield.

But the linear part of Fig 2.5 shows that the response of the
DCA derivative depends on the ADAM quantity, even when the
ADAM is in excess. The same behaviour was shown when the DCA
quantity was small enough to assure an excess of ADAM. The
hypothesis that one mole of ADAM will react with one mole of
DCA is contradicted. The yields of derivatisation will depend
on the absolute quantity of ADAM in the mixture . This can not
be explained by an uncomplete synthesis of ADAM, because the
FT-IR measurement gave a good yield.

The measurements of the DCA derivatisation however allow to
determine the capacity of ADAM to bind to DCA. We can say that
for 230 nmole of ADAM ideally formed in the synthesis mixture,
63 nmole of DCA represents the overdose. This means that in
order to derivatise 1 mole of DCA, ± 3.5 mole of ADAM will be
needed.

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2.3.3   Quantitative analysis of DCA

The calibration curve plots the peak area against the con-
centration of DCA (Fig 2.7). The linearity was tested in the
range of 5 and 33 ng, the correlation coefficient (0.992) was
good.

Fig 2.8 shows the variability of the retention time. The peak
area has been calculated by multiplying the peak height with
the half height width. However this measurement was
practically difficult and not accurate, and introduced there-
fore an additional factor of variability of the response.
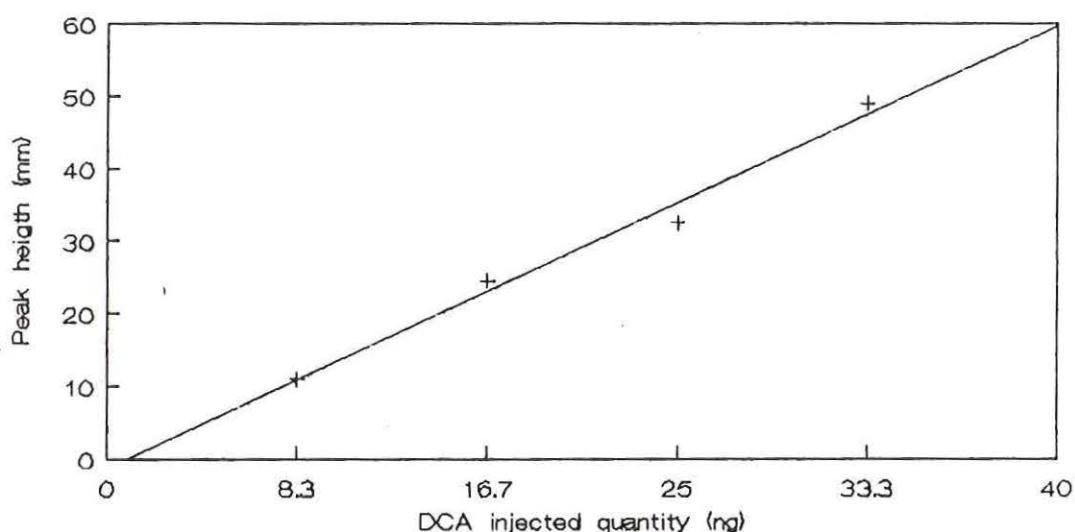
Figure 2.7 : Calibration of DCA; derivatisation in 300
μl.

The repeatability of the injection in the HPLC system was
tested by injecting 12 times an identical solution containing
2.5 ng of DCA derivative : the coefficient of variation was
3.4 %. These results, also presented in Fig 2.8, show also the
good stability over 12h of the derivatives under the
conditions of the Sample Injection processor, which allows
overnight analysis.

Precision of DCA derivatisation was tested by measuring the
peak area of identical DCA standard solutions derivatised 5
times, at a level of 2.5 ng DCA injected. The coefficient of
variation was 3.6 %.

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The stability of the derivatives in the derivatisation mixture was tested by measuring the response of the same derivatives solution, after several days. The DCA derivatives were stable for at last one week at room temperature in dark, but degradation occured when exposed to the light.

Figure 2.8 : Repeatability of the derivative injection and measurement; same solution of DCA derivative (2.5 ng/injection).

2.4 CONCLUSION

The derivatisation procedure described here, using ADAM syn-
thesised in situ, was satisfactorily reproducible for the
tested acid DCA. The fluorescent response of the derivative
was proportionally linear to the DCA concentration in the
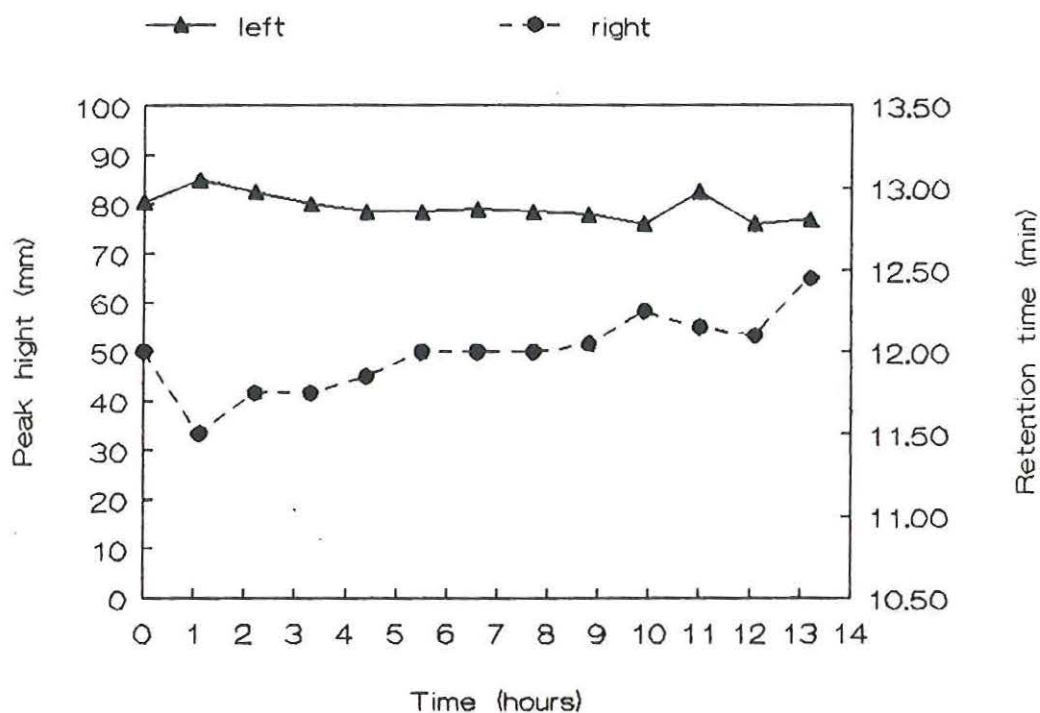derivatisation mixture.
The reactives still present in the synthesis mixture were
proved not to destroy the DCA, since the response was
increasing with increasing quantities of the ADAM synthesis
mixture.

However, the reliability of the derivatisation was not quanta-
tively good because the response of derivative was increasing
with the amount of ADAM. If the synthesis of ADAM in the in
situ mixture was proved to be sufficient, only 25% of the
theoretically available ADAM seemed to react with DCA. This
means that in order to derivatise the acid totally, a large
excess of ADAM must be present. The ADAM derivatisation was
reliable for a DCA concentration of 25 μg/derivatisation.

The stability of the reagent solutions for the synthesis of
ADAM was found to be limited. Within two months, many interfe-
ring peak appeared in the ·chromatograms, and a clean-up of the
derivative was necessary.

To assure a good synthesis of ADAM, either the stability of
the reagent solutions must be checked or the ADAM synthesis
yields must be controlled regularely. This can be done by
checking the derivatisation capacity of the ADAM.

For these reasons, the use of a internal standard was needed,
and is described below.

3 EXTRACTION OF OKADAIC ACID AND USE OF AN INTERNAL STANDARD

3.1 INTRODUCTION

To be used as a fluorescent labeling reagent for okadaic acid, the 9-anthryldiazomethane must offer a sufficient specificity. The method of derivatisation has to be reliable and easily applicable. In the former chapter, the derivatisation with ADAM synthesised in situ has been proved to be sufficiently sensitive and reproducible. However, to allow good quantification of the analyte, the derivatisation yield has to be checked, since the response of the derivative was found to be a function of the available amount of ADAM.
Furthermore, variations in the derivatisation yields due to the sample matrix can lead to errors and must therefore be verified.

So we developed the use of an internal standard for the determination of OA. This standard should be a carboxylic acid reacting with ADAM; its derivative should have the same behaviour during the clean-up procedure and it should be possible to separate them on the same HPLC system. The internal standard will be added to samples extracts or OA standards just before derivatisation.

The utility of this internal standard should be twofold:

- first, to check day to day derivatisations. Because a freshly prepared ADAM synthesis mixture is used each day, the internal standard will allow to check the progress of the ADAM synthesis, and the quality of the reagent solutions. The fluorescent response of the internal standard could then be used for a long term control.

- second, to check the derivatisation yield from one same ADAM synthesis mixture as well as the clean-up of the derivatives after reaction. The use of an internal standard will make the quantification of OA independent of factors having an effect on both the derivatisation yield and the clean-up recovery (as the internal standard should have the same reactivity with ADAM as OA).

In this chapter, the use of DCA as a internal standard will be examined. Then, it has been used to test the extraction method for OA according to Lee et al.(1987).

3.2 METHOD

As the ADAM synthesis mixture and ADAM derivatives are unstable when exposed to light at room temperature, all preparative operations have been done in a room shielded from day light, with subdued lighting.

The synthesis in situ of ADAM as well as the HPLC conditions for measurement are described in the former chapter.

3.2.1 Reagents

- okadaic acid standard: Promochem, X132 (C.N. Schmidt, Amsterdam), 100 μg/ml in dimethyl formamide.
    stock solution: 10 μg/ml in methanol, stored at 4°C in the dark, stable for one year.
    working solutions: from 10 to 0.5 μg/ml in methanol by diluting the stock solution.
- caprylic acid reference standard : Fluka # 21639
    stock solution : 10 mM in 95% ethanol

- ADAM synthesis mixture and DCA solutions, see former chapter.

3.2.2 Apparatus

Sorvall Omni-mixer, Dupont Intrument, with 50 ml metal baker.
- Seppak silica cartridge, Waters # 51900.
    The columns were placed in a dessicator after the original packaging was opened, to prevent water absorption.
- Seppak C-18 cartridge, Waters # 51910.

- HPLC system : see former chapter.

3.2.3 Sample preparation

The blank samples were digestive glands (hepatopancreas) of mussels bought on the market and supposed to be free of toxins. The dark brown glands were taken out from fresh or cooked mussels, weighted and stored frozen at -4°C.

For cooked material, the fresh mussels were allowed to cook for 7 to 10 minutes. After cooling, the digestive glands were removed from the meat.

For dried material, the entire fresh or cooked glands were lyophylised, and reduced to powder, which was stored in the dark at room temperature.

A contaminated sample, (confirmed by rat test) was obtained from the RIVO, IJmuiden (Netherlands Institute for Fish Research). This sample was cooked, lyophylised and reduced to powder at RIVO.

3.2.4 Extraction

For extraction, 2 g of wet sample or 0.5 g of dried sample was used. In a metal baker adapted to the Omni-mixer, 8 ml of methanol 80% (v/v) was added, and homogenised for 5 minutes. The homogenate was centrifuged (3500 rpm) for 10 minutes. An 2.5 ml aliquot of the supernatant was transferred to a test tube, and washed 2 times with 4 ml hexane.

For the C-18 clean-up, 0.625 ml of the methanolic extract was added to 0.375 ml water (Milli Q), in order to obtain a methanol/water ratio of 50/50 (v/v). An aliquot of 0.5 ml was transferred to the C-18 cartridge column which was first conditioned with 5 ml methanol, and then with 5 ml methanol 60% (v/v). After loading the sample the column is eluted with 3 ml of methanol 60% (v/v). Total eluate was collected and extracted with chloroform as described further.

For the samples without C-18 clean-up, the methanolic extract was added to water in order to have a methanol/water ratio of 60/40. The resulting mixture was then extracted as follows.

A 3 ml aliquot of the methanolic solution was extracted two times with 4 ml chloroform. The combined chloroform extracts was evaporated at 50°C under nitrogen flow. The residue was dissolved in 300 μl methanol, containing the internal standard DCA (0.1 or 0.05 μg/100 μl); 100 μl was used for derivatisation.

3.2.5 Derivatisation with ADAM

The derivatisation with ADAM was carried out in a glass micro-tube (300 μl vial insert for WISP vials). 100μl of the ADAM synthesis mixture was added to 100μl of the extract solution. The solution was mixed, and the vial is placed in thermo-block and allowed to stand one night at 50°C.

3.2.6 Derivative clean-up

An aliquot of 100 μl of the derivatised mixture was pipetted in a pointed glass tube, and the solvent was evaporated to dryness under nitrogen at 50°C.

The silica cartridge column was conditionned with 5 ml hexane-/chloroform 50:50 (v/v). The derivatisation residue was transferred with 3 times 1 ml of hexane/chloroform 50:50 (v/v), to the cartridge, and the eluate was discarded. The column was washed with 5 ml of the same solvent, and then with 4 ml chloroform.

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The derivatives were eluted with 5 ml chlorofom/methanol 95:5
(v/v), and collected in a pointed test tube. After evaporating
the solvent to dryness under nitrogen at 50°C, the residue was
dissolved in 200 µl methanol, and transferred in a 300 µl glass
vial, tightly stoppered for storage in darkness until injection
into the HPLC system.

3.3 RESULTS AND DISCUSSION

3.3.1 Use of internal standard for the OA determination

3.3.1.1 Derivatisation of okadaic acid

The rate of OA reaction with ADAM has been verified at 50°C
(Fig 3.1). The time curve for the OA derivative yield followed
the same shape as for the DCA derivative, reaching a maximum
plateau after 10 hours.
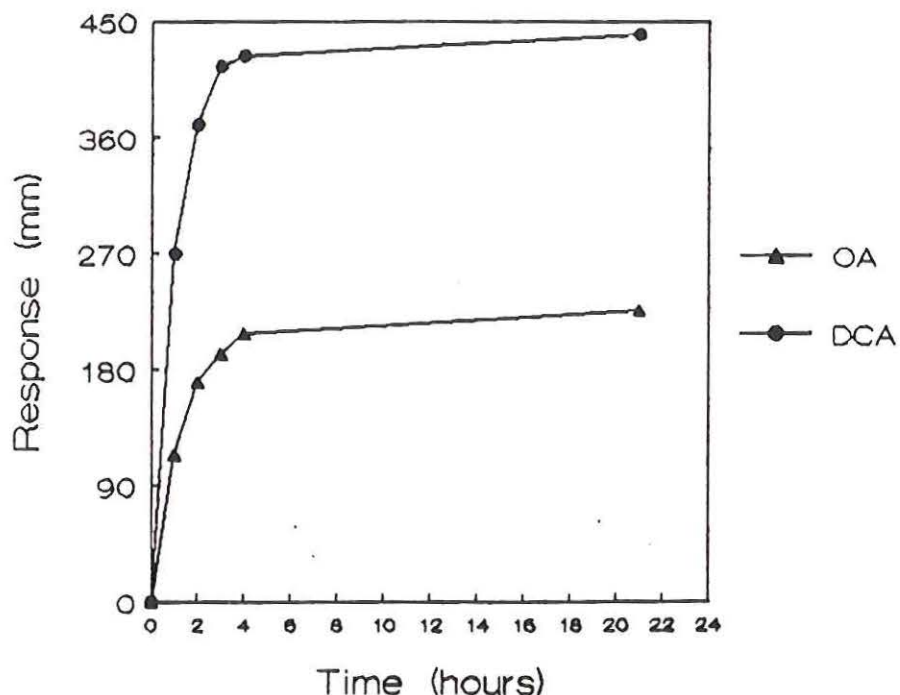
Figure 3.1: Derivatisation rate of OA and DCA, 1 µg of
each compound were derivatised in 3 ml (without clean-
up).

Under the chromatographic conditions, the separation of both
derivatives was good. The acetonitrile/water system as mobile
phase gave a good separation. Typical chromatograms of ADAM
derivatives are shown in Fig 3.2.

OA
1,88 ng

DCA
0,625 ng
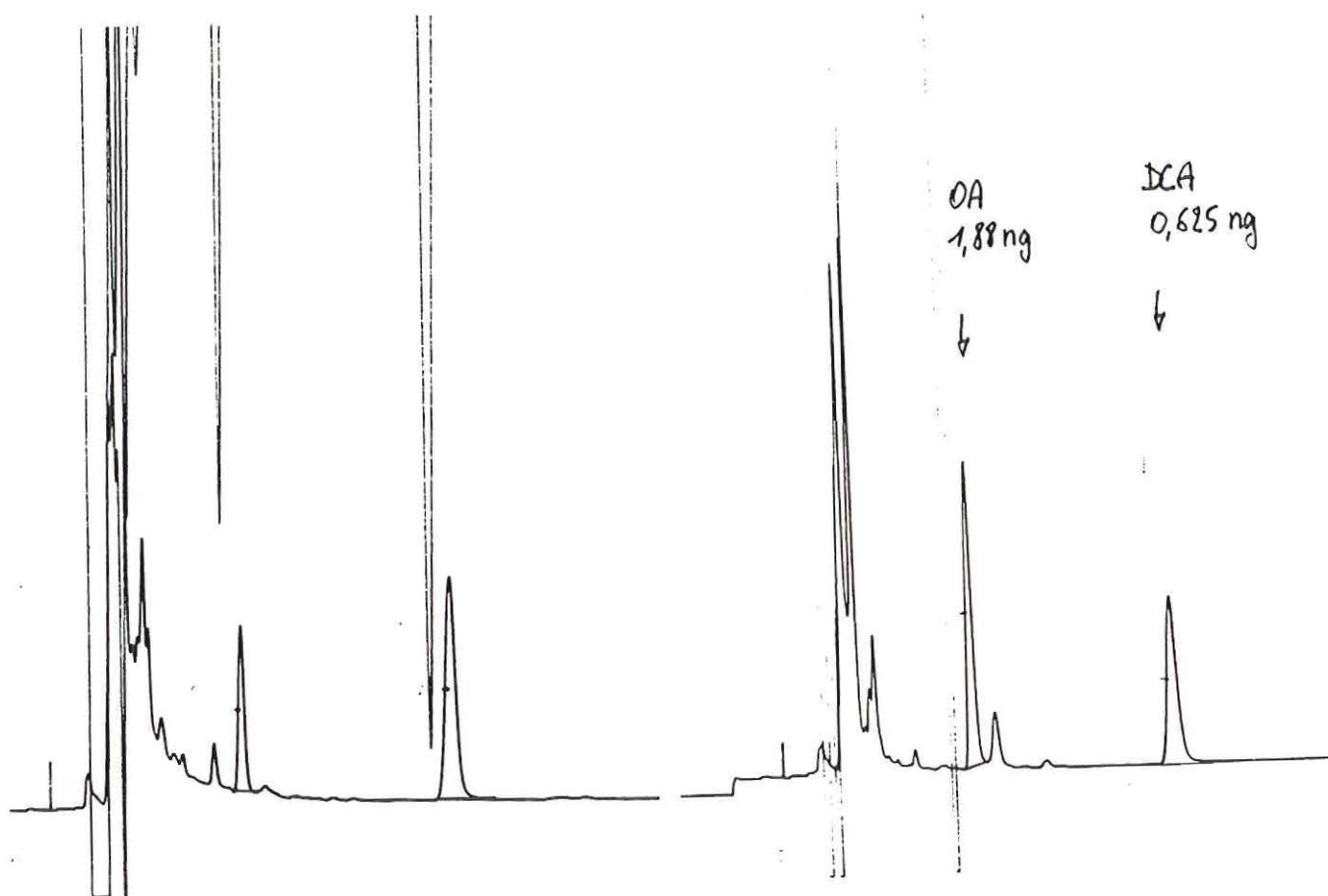
Mussel matrix spiked with
OA standard
(1µg/g wet hepatopancreas)

Standards of OA and
DCA

Fig 3.2 : Chromatogram of derivatised OA and DCA stan-
dard, with only silica clean-up.

3.3.1.2 Calibration curves

The fluorescence responses of DCA and OA standard solutions gave both a good linearity. When the peak areas are plotted against the amount of OA and DCA in ng injected (Fig 3.3), the ratio of the slopes a_{OA}/a_{DCA} is 0.456. Fig 3.4 shows the calibration lines obtained when the responses are plotted against the acid amounts in pmole injected. The ratio of the slope is then 0.93, since the ratio of the molecular weight of these two acids MW DCA/MW OA (392.5/804) is 0.489.

This shows that both components have the same equilibrium constant for the reaction with ADAM. Both derivatives have the same fluorescence intensity.

3.3.1.3 Silicagel clean-up

The silicagel clean-up was necessary after derivatisation for removing excess of ADAM and by-products of the derivatisation. This clean-up allowed the removal of a small peak eluting at the same retention time as the OA derivative. It also allowed to have a shorter HPLC analysis time and enhanced the life time of the HPLC guard column.

The clean-up was carried out on a small silicagel cartridge. The eluting pattern has been checked for high recovery. Without the chloroform wash, the recoveries were less variable, but this wash step was needed to clear the chromatogram from all other peaks derived from the matrix.

Fig 3.5 and fig 3.6 show the calibration plots constructed after silicagel clean-up. The linearity has been verified on a range of 0.05 to 0.15 ng derivatised. The good linearity of the OA/DCA ratio (Fig 3.7), allows thus the quantification of OA corrected for the derivatisation yield and clean-up recovery.
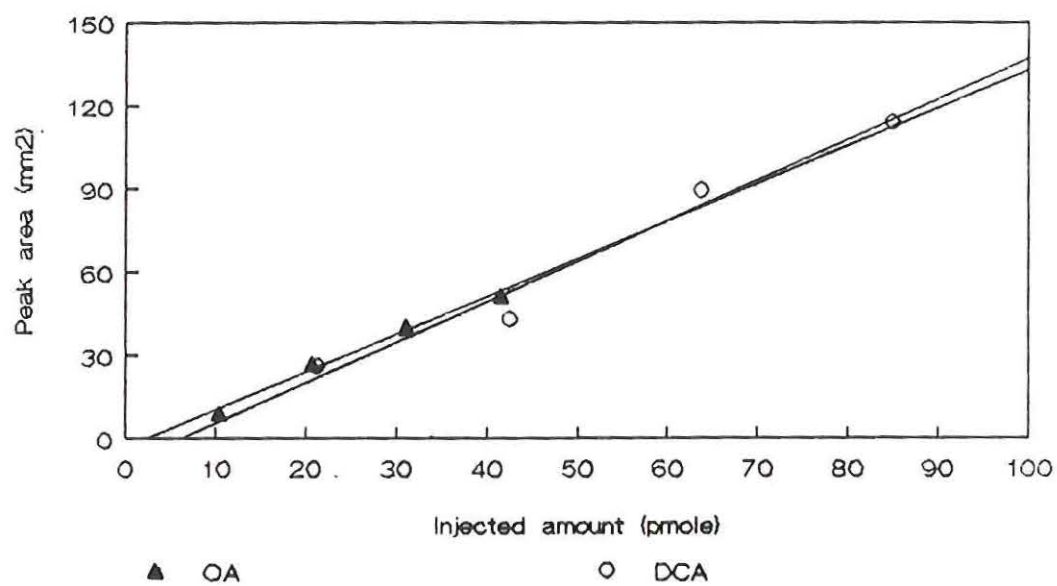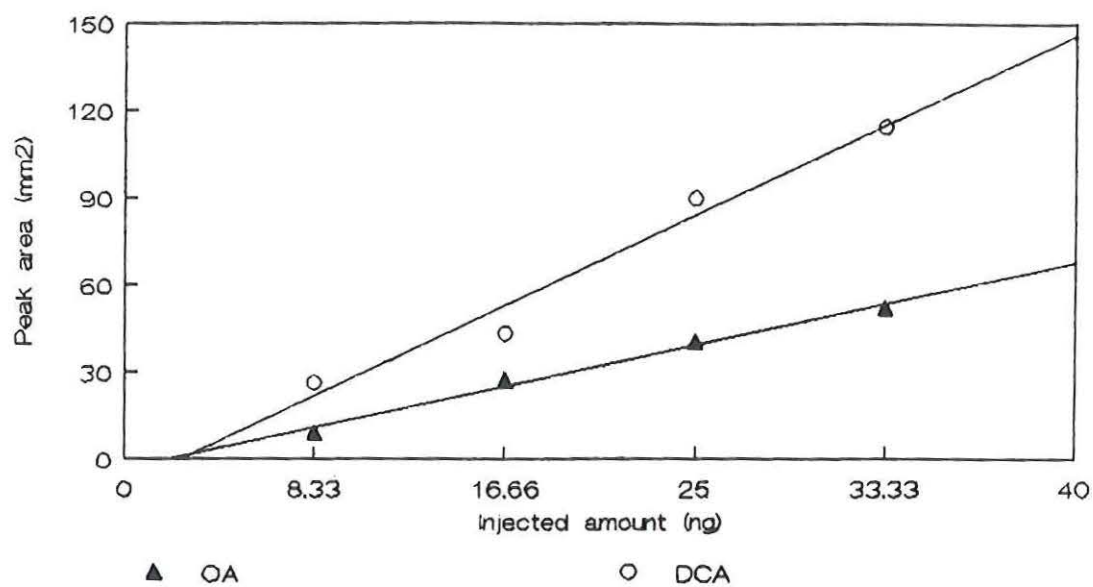
Figures 3.3 and 3.4 : Calibration curve of DCA and OA derivatives, standards were separately derivatised in a total volume of 300 μl.
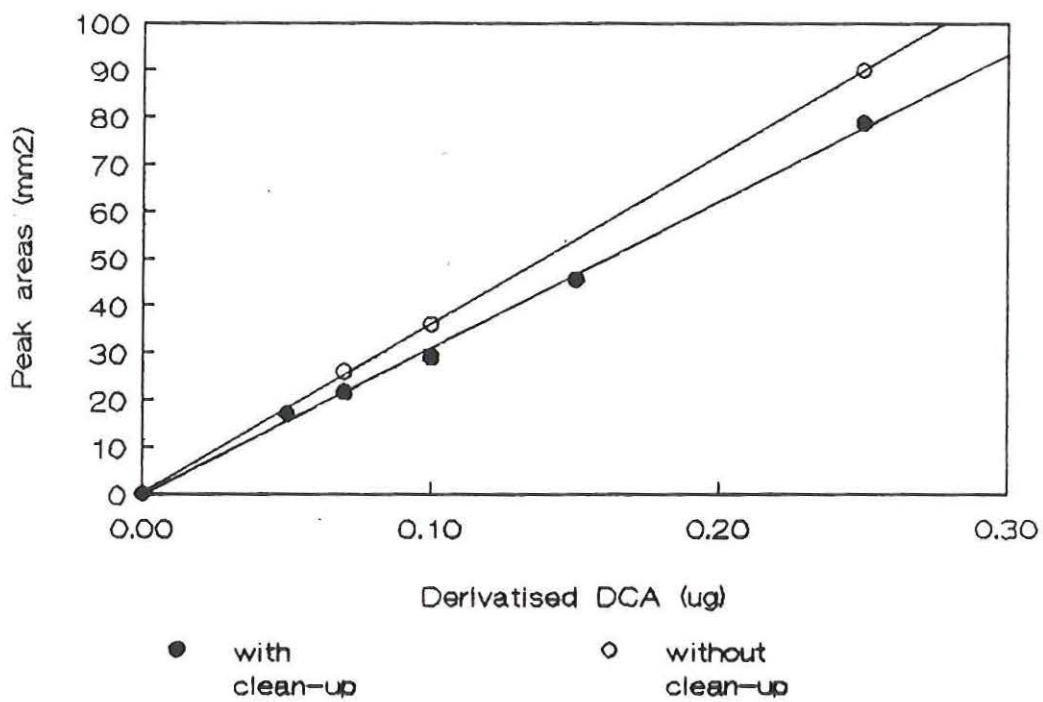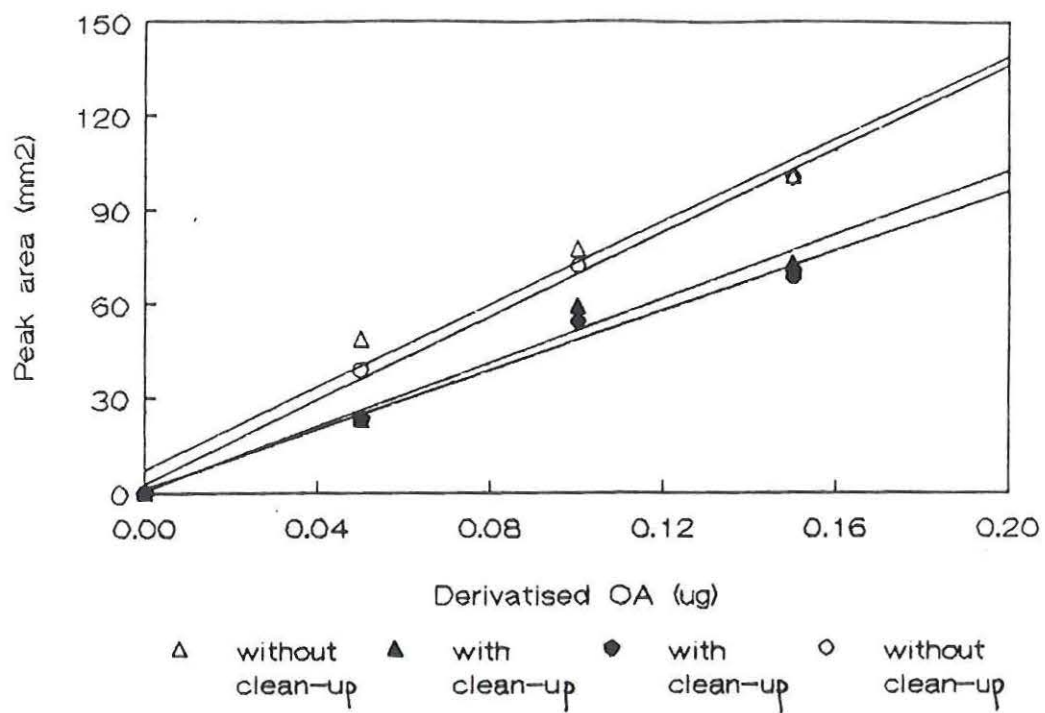
Figure 3.6 and 3.7 : linearity of the OA and DCA deriva-
tives after silica clean-up.
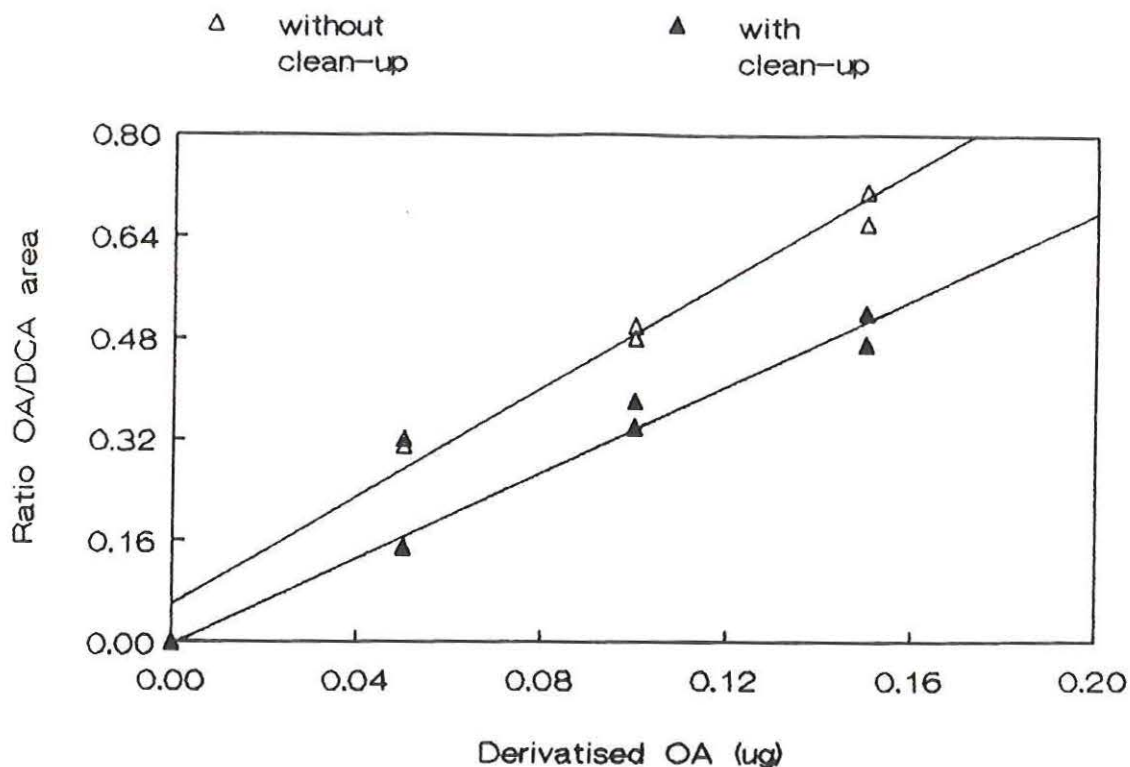
Figure 3.7 : Linearity of the OA response corrected for the DCA response; OA was derivatised with constant amount of DCA (0.125 μg).

3.3.1.4 Reproducibility and limit of detection

The precision of the analysis has been tested by measuring the fluorescence responses obtained from 8 different derivatisations of 0.05 μg of both DCA and OA, with silica clean-up. The coefficients of variation were 4.7% for DCA, 6.9% for OA and 5.0% for the ratio of OA/DCA peak area (Fig 3.8, 3.9).

At the sensitivity of the recorder used for the analysis of mussel samples, the noise was measured in a blank standard (at the OA-ADAM retention time). The limit of determination (10 times the noise) could reach the value of 0.5 ng OA injected. This means a theoretical limit of determination in sample extracts of 400 ng/g wet hepatopancreas or 0.1 μg/g dry matrix (following the extraction conditions described above). The lowest standard concentration tested was 0.063 μg OA/ml, giving 0.625 ng injected.

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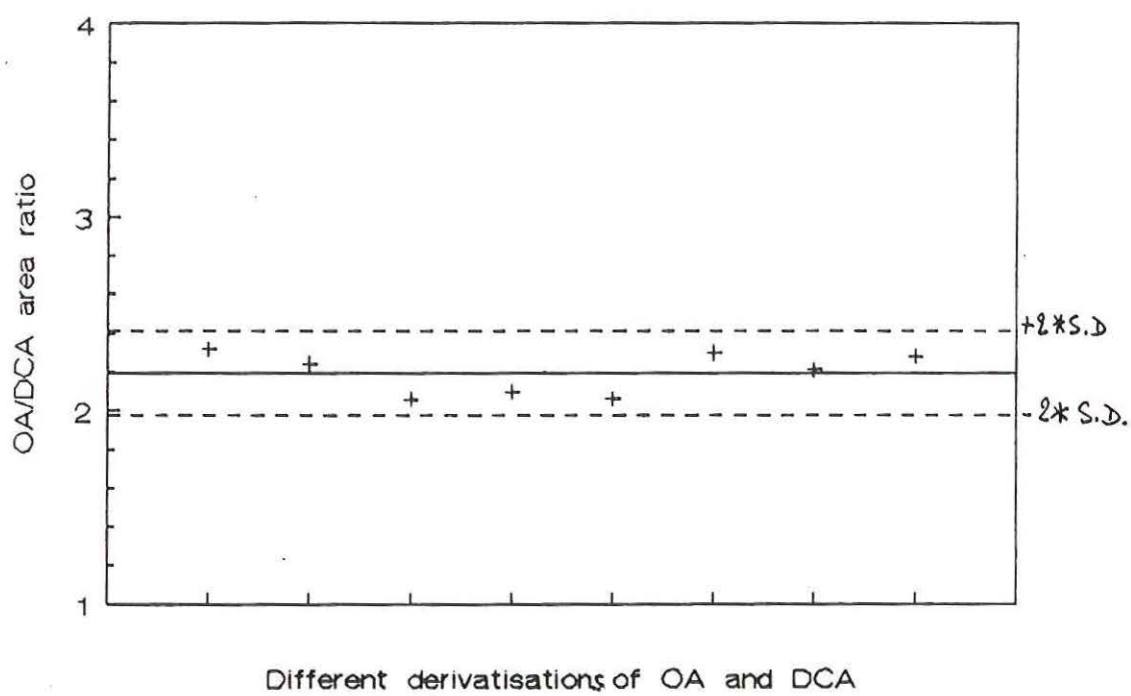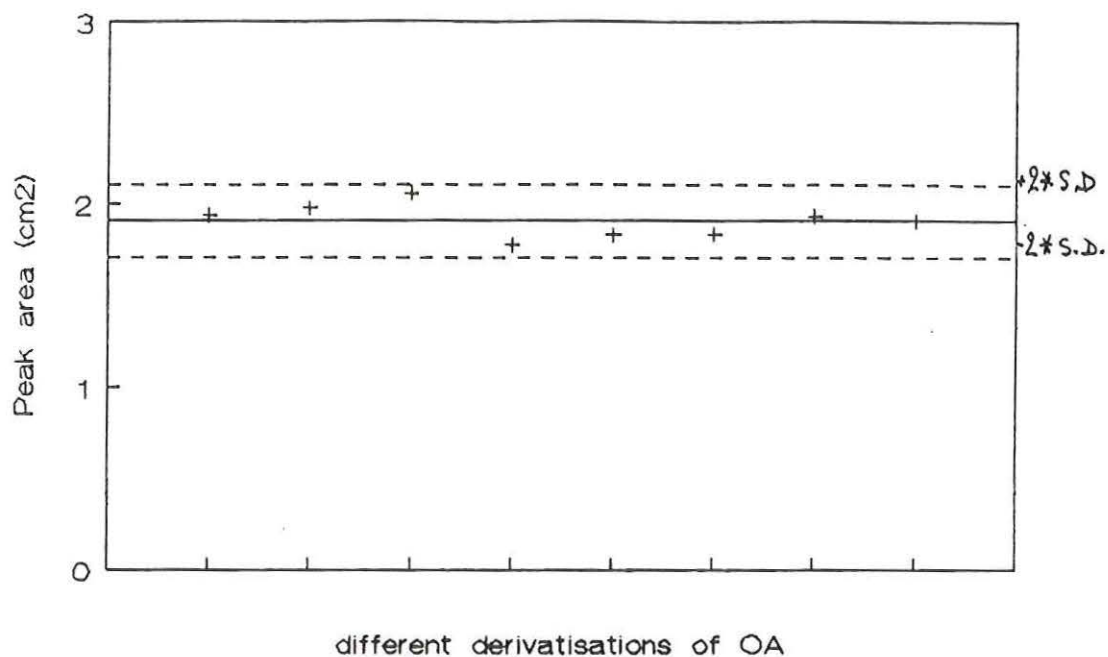
Figure 3.8 and 3.9 : Reproducibility of the OA and OA/DCA response. SD = standard deviation.

3.3.1.5 Conclusion

To be used as an internal standard, DCA should present some characteristics which have been checked:

-the DCA derivative is determined on the HPLC system, and its retention time is great enough to prevent all interferences.

-the DCA derivative has a behaviour on the silicagel column comparable to that of OA. The linearity of DCA recovery has been proved as well as for OA recovery. The respective recoveries were 85% and 75%.

-DCA reacts quantitatively with ADAM and the presence of this internal standard has no effect on the derivatisation of OA. The calibration curves have shown that both acids have the same reactivity with ADAM.

-DCA is stable in methanol for at least one year. The stock solution was derivatised simultaneousely with freshly made standard solution, and no significant difference has been observed. This allows long term control of derivatisation.

Since these conditions are fulfilled, the ratio of the peak area OA/DCA may be used to quantify OA. The practical applications of this internal standard will be :

- added to OA standard as well as to sample extracts, the absolute peak area of DCA derivatives will allow to check and quantify the derivatisation yields. The effect of various extraction conditions on the derivatisation can be seen. The absolute response of DCA allows also to take into account the non-systematic errors that could occur in a set of analysis losses by manipulation, volume change by solvent evaporation, etc.).

-the ratio of OA/DCA peak area may be used to plot the calibration curve to quantify OA. So the recoveries of both derivatisation and silicagel clean-up are directly taken in account. This means that OA responses will be corrected for incomplete derivatisation.

-given the good stability of DCA in stock solution, the ratio of the OA/DCA response will provide a measurement of the stability of OA standard solutions.

3.3.2 **Extraction of OA from mussel matrix**

The procedure of extraction is the method of Lee et al. (19-87), with the following major modifications :

- for recovery study, adjustment of the pH of the methanolic extract has been incorporated, as well as an additional clean-up on C18 cartridge before the chloroform extraction and derivatisation.

- the internal standard DCA is added to the samples before the derivatisation, and allows the control of this reaction and clean-up step.

The table 3.1 summaries the extraction procedure.

Tab 3.1 : SIMPLIFIED FLOWCHART OF THE TOTAL PROCEDURE

Wet sample   0.5 g dry hepatopancreas
2 g   (corresponding to 2 g wet)
                  + 8 ml MeOH 80%

                  mixed and centrifuged (5 min, 3500 rpm)
                  (sometimes addition of NaHCO$_3$ 0.75 %
                  w/v to supernatant)

0.625 g   2.5 ml aliquot of supernatant

                    2 * 2.5 ml hexane washing

0.156 g   0.625 ml aliquot of methanolic phase
                  or + 2.375 ml MeOH 55%   or + 0.375 ml water
                                  0.5 ml on C-18 column*
                                  elution with 3 ml MeOH 60 %
                  2 * 4 ml chloroform extraction
                  evaporated, N2 at 50 °C

                  +300 μl DCA 0.5 μg/ml in MeOH
                              (ultrasonication)
0.052 g(1/40) 100 μL aliquot derivatised
                  with 100 μl ADAM solution**
                  one night, 50 °C

0.026 g   100 μl aliquot dried and used for
                  silica gel clean-up***
                  elution with 5 ml chloroform/MeOH 95/5
                  evaporated

                  taken in 200 μl MeOH (ultrasonication)

1.3 mg(1/1600)   10 μl injected in HPLC
----
* C-18 cartridge : Seppack nr 51910
                  conditionned 5 ml MeOH
                          5 ml MeOH 60%
                  addition of 0.5 ml sample
                  eluted with 3 ml MeOH 60%
** ADAM solution : synthesised in situ,30 min in ethylacetate
                  500 μl of 6.9 mM 9-anthraldehyde hydrazone
                  500 μl of 6.9 mM N-chlorosuccinimide
                  500 μl of 69 mM quinuclidine
*** Silicagel cartridge : Seppack nr 51900
                  conditionned 5 ml hexane/chloroform 50/50
                  addition of sample in 3*1 ml of same solvant
                  washed with 5 ml same solvant
                          4 ml chloroform
                  eluted with 5 ml chloroform/MeOH 95/5

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3.3.2.1 Effect of pH and matrix compounds.

The effect of sample extract on the derivatisation has been pointed out by comparing the fluorescence response of the standard solution DCA or OA, and the response of these acids when derivatised with sample extracts. In matrix, the OA derivatisation yield was significantly lower, and could reach the extreme value of 23% of the standard response. It was also observed that this yield varied with the various matrices that have been used.

This decrease of response could be due to the competition of carboxylic acids from the matrix that can also react with ADAM; this could also be due to the effect of various pH or ionic composition affecting the derivatisation reaction. The acid must indeed be present under an acidic form to react with ADAM (Takatsuki et al., 1986).

Furthermore the pH of the initial extraction mixture could also theoretically have an effect on the partition of OA in the extractions occuring in the protocol, (hexane, methanol (80%) different phase and chloroform-methanol (60%)). The effect of the pH was first examined, since different matrices showed a variable pH when homogenized in methanol 80%. The pH of the methanolic extract solutions were below 7 once the sample was homogenized with the mixer and this pH varied between 4.5 and 6.5 from one matrix to another.

The effects on both the derivatisation and the extraction recovery have been checked. For that, OA has been extracted from methanolic solutions containing caprylic acid (C8) used as a model for the sample matrix. The pH of the solutions have been adjusted by adding NaOH 0.1 mM and measured by pH-meter. The internal standard DCA was added after the extraction procedure, just before derivatisation. The extraction was carried out following the protocol in table 3.1 and the results are expressed in relative responses in tab 3.2.

SOLUTION composition	pH	DCA response	OA/DCA response	OA response
MeOH 80% + NaOH	6.8	set at 100%	set at 100%	set at 100
	7.6	105%	107%	112%
MeOH 80% + C8 0.2 mM + C8 0.2 mM and NaOH	5.5	22%	73.5%	16%
	7.6	46%	87%	41%

Tab 3.2 : Effect of pH and fatty acid on the derivati-
sation yield and the extraction recovery (relative re-
sults).

The results show first that the responses of DCA and OA are
similar. It can be concluded that the OA recovery is very
high, and that DCA reacts with ADAM in the same way as OA.
This allows the use of DCA as an internal standard, even under
various conditions of pH.
Using DCA as internal standard, the results show further that:
- addition of C8 acid and various pH influence mainly the
  derivatisation process (DCA response), and only slightly the
  extraction recovery (OA/DCA response).
- the main influence on the derivatisation yield is caused by
  the competition of the C8 acid for reaction with ADAM. The
  effect of alkaline pH is more pronounced when C8 is present
  in the derivatisation solution.
This will be discussed in the next chapter (3.3.2.2).

The fatty acid C8 has been used as a model of the matrix
extract. Even if it doesn't precisely correspond to the ma-
trix, the results show that a carboxylic acid in high concen-
tration affects mainly the derivatisation yield. Although the
use of the internal standard allows still good accuracy of the
OA determination, the limit of detection of the method will be
higher.

3.3.2.2   C-18 clean-up

Reverse phase column chromatography was introduced in the
extraction protocol in order to remove as much as possible the
matrix compounds before the derivatisation. This should allow
a better and more constant derivatisation, since the other

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carboxylic acids represent a competition for reaction with ADAM. Luckas et al. (1991) have proposed a clean-up on a C-18 cartridge. The methanolic solution was transfered on the C-18 column and eluted with methanol 60%. The chloroform extraction was then carried out, like in the classical protocol.

The pattern of elution was examined for C-18 cartridge columns. The recovery was optimal following the procedure present in table 3.1. The recovery of OA was enhanced when the column was previously conditioned with pure methanol. Because the first discarded eluate was found to contain a great amount of OA, a sample volume of 0.5 ml was applied to the column and nothing of the eluate should be discarded!
In these conditions, the recovery found was 85% ± 3.7 (n=4). The clean-up however introduced a higher variability in the results.

The effect of the washing step with hexane prior to the C-18 column on the recovery of the C-18 column was examined. OA standard was added in 3 ml methanol 80% at a level of 0.05 μg/ml. These solutions were washed with 2.5 ml, 4 ml hexane or not washed at all. An 0.5 ml aliquot was transferred on the C-18 cartridge, previously conditionned with methanol. Comparison of responses obtained with various washing showed that the volume of hexane had no effect on the OA recovery through C-18 clean-up.

The effect of the additional C-18 clean-up on the total determination was examined at pH 5 and 8 by extracting a contaminated lyophylised sample. Table 3.3 shows the relative recoveries that were obtained under the different extraction procedures. 0.5 g of dried sample were extracted two times with methanol 80%; the natural pH was 5.5. The sample was extracted two other times ; the pH of the methanolic solutions after homogenisation were adjusted to pH 8 with HCO_3^- 3 mM. The C-18 clean-up was carried out after 1 ml of sample applying on the column.

SOLUTION	alkaline addition	C-18 clean-up	DCA response	OA/DCA response
standard	-	-	set at 100%n=3	--
sample 1	-	-	23% (n=2)	set at100%n=2
2	-	+	52% ±3.7(n=3)	18% ±6 (n=3)
3	+	-	46% ±8 (n=4)	78% ±5.5 (n=3)
4	+	+	85% (n=2)	97% (n=2)

Table 3.3 : effect of pH and clean-up on the derivatisation of DCA and extraction of OA (expressed in relative response) when extracting a contaminated sample: DCA was added before derivatisation (0.05 μg/100μl).

The effect on the derivatisation is shown by the internal standard response (fourth column). It can be seen that:

-with an acidic extract, the derivatisation yield is the lowest, but is well enhanced by the C-18 clean-up. Since the pH of the samples were still acidic (pH 5.5) after the C-18 clean-up, the higher response can be explained by the fact that the C-18 removes the acids in competition with OA for ADAM.

- both C-18 and addition of bicarbonate enhance the response of DCA. This proves that the derivatisation depends on both parameters, the form of the acid and the competition of other acids coming from the matrix.

The highest derivatisation yields were obtained with an alkaline extracted solution cleaned up on the reverse phase cartridge. Furthermore the chromatograms were much cleaner with this protocol.

The effect on the extraction is shown in the fifth column of tab 3.3, calculated with the response ratio OA/DCA. The recoveries are expressed in relative values. It can be seen that:

- the recovery of OA through the C-18 clean-up is drastically higher at an alkaline pH.

-only a small difference is found between extraction without C-18, at alkaline pH and at natural pH (without bicarbonate).

These results confirmed the effect of pH on the OA chemical form. Because the OA is similar to the antibiotic ionophore Monensin, it could be assumed that the toxin can exist as a sodium salt, which forms a stable lipophilic complex. Since the sample matrix contains sodium ions, the OA can occur under this salt form in methanol 80%. Then, in a acidic solution, OA would be present under a free acidic form; in a high ionic and slightly alkaline solution, OA would be as a lipophilic complex.
These chemical form changes will have consequences on the OA recovery. First, at a pH 5 (sample 1 in table 2) the chloroform allows a good extraction of OA as in acid form. But these conditions are also favourable for the extraction of other acids coming from the matrix and then will lead to lower derivatisation yields, as it has been observed. On the other hand, at a pH 8 (sample 3 in tab. 2), the OA recovery is slightly lower. This is in agreement with Allenmark et al. (1990). They have shown that at pH 7.1, 80% of OA can be extracted in chloroform, as an ion pair form. This alcaline pH presents thus a lot of advantages, e.g. less co-extracted compounds giving better derivatisation yields, higher recovery through C-18 clean-up, better chromatograms. But with the high pH in the final methanolic extract, OA is present in an ion

form. This will lead theoretically to a worse derivatisation yield, because the acid must be in a free form to react with ADAM. The extract could then be acidified just before derivatisation.

Further analyses are needed to determine whether the presence of ions in the sample interferes with the retention of OA in the extractions, hexane wash or chloroform extraction.

In conclusion, the C-18 chromatography should be done only with a adjusted alkaline pH. The sensitivity of the analysis will be higher when the procedure includes a C-18 clean-up because the derivatisation yield is higher. Another advantage of the clean-up is that the chromatograms are much cleaner and free of compounds derived from the matrix. The disadvantage of the additional clean-up is the greater variability of the results.

3.3.2.3 Recovery of extraction

The average recoveries were determined on matrix samples spiked with OA standard. Cooked and lyophylised hepatopancreas of uncontaminated mussels were used as blank samples. The water content of the original matrix was 72% (1g wet = 0.28 g dry). About 0.5 g of dry hepatopancreas were spiked with OA at levels of 2, 1 and 0.5 μg/g wet hepatopancreas. From each spiked sample, four methanol extractions were carried out. A blank chemicals with OA corresponding to a level of 0.56 μg/g wet hepatopancreas was extracted simultaneously. A blank matrix (no OA added), was also extracted and showed no interfering peak at OA retention time. The reference standards of OA were derivatised and cleaned up for calibration at concentration corresponding to 1.92 and 0.96 μg/g wet samples.

The recoveries of the spiked matrix and the blank matrix are shown in table 3.4. The linearity of the response of OA is good (Fig 3.10 and 3.11). It can be seen that the OA/DCA ratio gives a lower variability in the results. This is not due to injection or recorder variation since it has been proved to be very low (3.3%). The manipulation variability occurs during the clean-up, and the more probable source of variation is the step of dissolving the dry residue in the small methanol volume.

SAMPLES	OA level (µg/g wet)	Recovery from OA response	Recovery from OA/DCA resp.
Spikes 1	2 µg	76% ± 6.4	71% ± 5.6
2	1 µg	75% ± 4.1	87% ± 4.2
3	0.5 µg	69%	89%
Blank	0.56 µg	82% ± 14	84% ± 3.1

Table 3.4 : recovery of OA in spiked matrix. Results are mean values ± standard deviation, of 4 different methanol extractions (except spike 3, extraction was carried out in duplo).

In another experiment, the determination in the contaminated dried sample was carried out with standard addition, at an adjusted pH of 7.5. OA was added to 0.5 g of dried sample at levels of 1.34, 2.66 and 4 µg/g dried sample. The extraction was performed with and without C-18 clean-up. The plotted responses are presented Fig 3.12 and 3.13, where the linearity was very good.

The recovery of OA through the complete extraction procedure without C-18 clean-up was this time 54% (47% with C-18). This is quite lower than this obtained with the other matrix. This confirmed that the recovery of extraction depends on the pH and then on the nature and composition of the matrix.
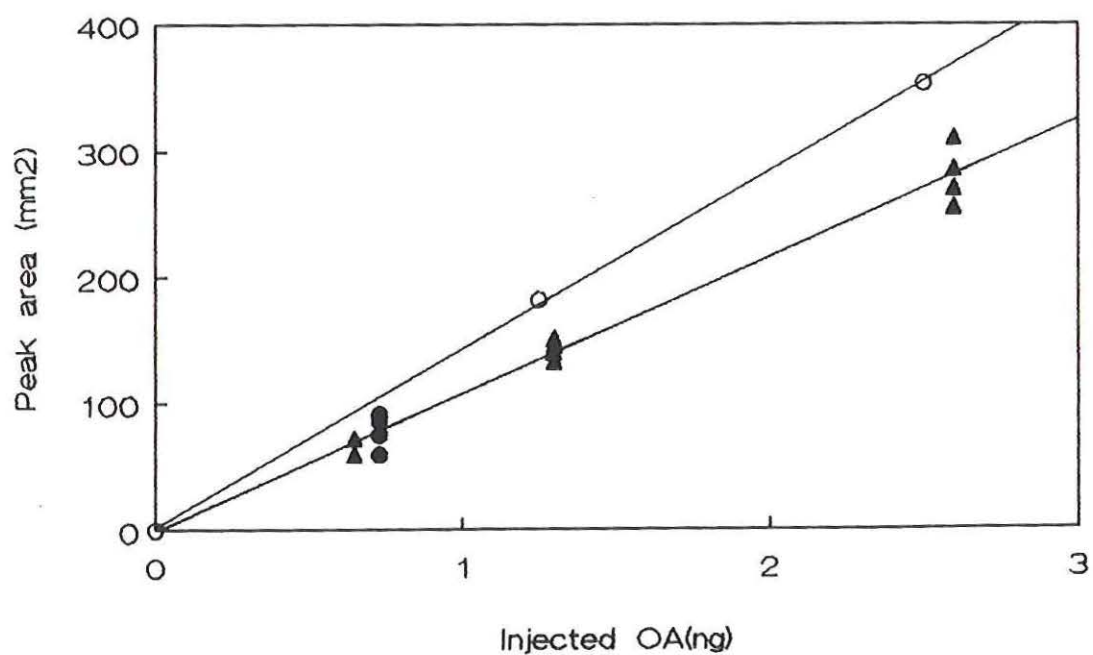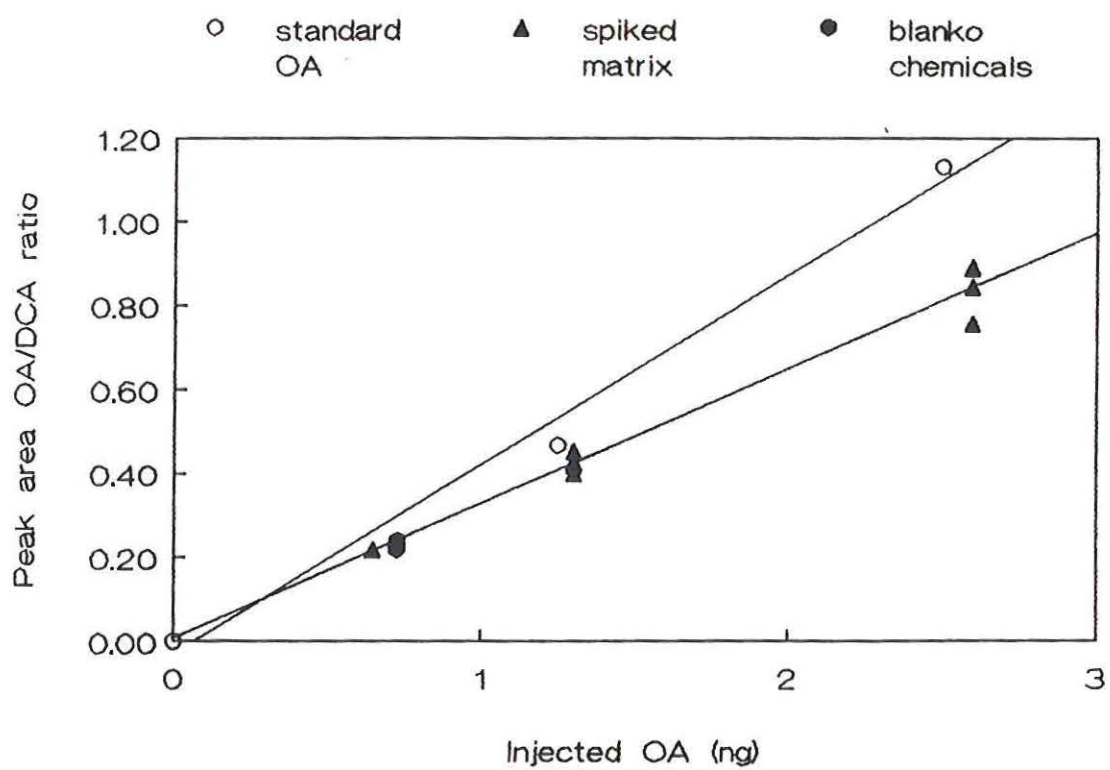
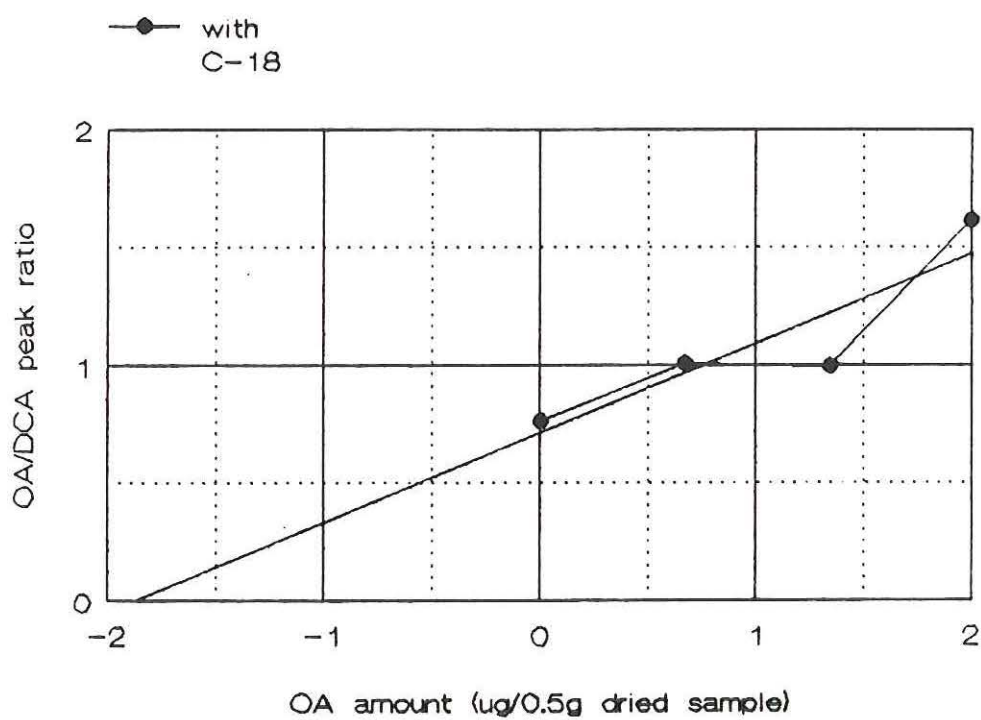Figure 3.10 and 3.11 : Recovery of OA in matrix spiked with OA standard;
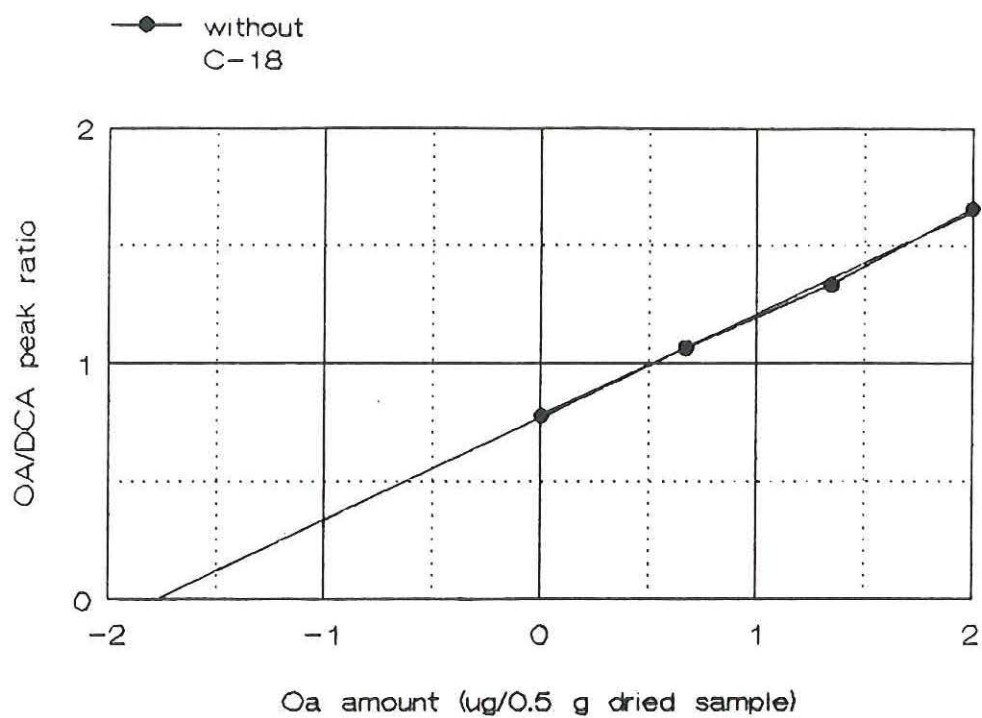
Figure 3.12 and 3.13 : Extraction of OA in a contaminated sample with OA standard addition at levels of 1.34,, 2.66 and 4 μg/g dried hepatopancreas.

3.3.2.4 Stability of OA derivative

Stability of OA and DCA derivatives has been checked by deri-
vatising the same solution of OA and DCA (0.1 μg/100 μl each)
8 different times with the same ADAM synthesis mixture. These
solutions were injected directly after silica gel clean-up and
after 11 days standing in the dark. The variability of the
responses was greater, but the error intervals overlap each
other (Fig 3.14). It can be concluded that the derivatives
were stable within at least 10 days.

figure 3.14 : Stability of OA and DCA derivatives, stored in
dark. SD= standard deviation.

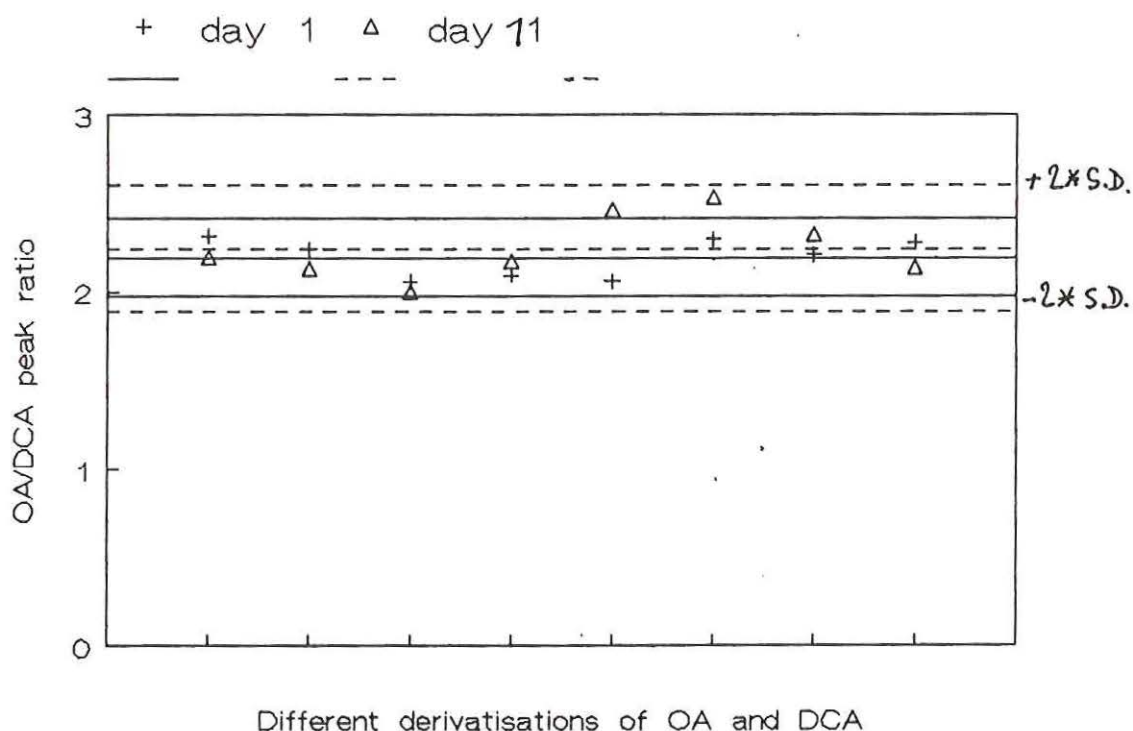## 3.3.2.5 Conclusion

The deoxycholic acid has found to be a excellent internal standard; this acid is stable, the chromatographic behaviour of the ADAM derivative is close to that of OA derivative. Added to standard and samples, DCA allows to correct the OA response for proportional and systematic error due to uncomplete derivatisation, as wel as for random errors occuring in manipulation.

With the use of this internal standard, the determination of OA with ADAM synthesised in situ was proved to be reliable and reproducible.

However the determination of OA in samples was found to be dependant on the matrix characteristics. Examination of the recovery of OA extraction from a sample matrix, showed that the pH, ions addition and additional clean-up have an effect on both derivatisation and extraction. The highest OA response was found with an extraction procedure at pH around 7.5 including a C-18 column chromatography of the methanolic extract.

Compared to the method of Lee et al. (1987), the procedure used here has a lower dilution of the extract during the preparation. 2 g of wet hepatopancreas (or 0.5 g of dry hepatopancreas) are extracted, and ca. 1/40 of this sample is derivatised. This means that 0.052 g of wet sample are derivatised. This allows to reduce the limit of detection.

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4 OVERALL CONCLUSIONS

Problems of OA determination appeared not to lie in the esterification with ADAM but rather in the extensive sample preparation. Extraction recoveries of OA was satisfactory but variable. Especially the type of the matrix gave different recovery. Several factors influencing the extraction of OA have been pointed out. They should be controlled and normalised in order to make the determination independant from the quality of the sample. Therefore we suggested an extraction with a slightly alkaline buffer (pH 7.1 to 8). Other factors, like the degree of degradation of the matrix tissue etc, should be investigated in order to establish a protocol that leads to a normalised extraction procedure in various mussel samples and other shellfish samples.

The losses of OA during the extraction should be further investigated, in relation to the lyophylisation and the cooking of the matrix, and also for possibilities of enzymatic oxidation in the sample.

The use of DCA for internal standardisation is strongly recommended for control of the derivatisation yield. Furthermore this component can be used for intercalibration between several laboratories : DCA is easily available as a stable solid standard, and can be used to check and compare the OA reference standard from different sources.

The method of Lee et al, applied with the ADAM synthesised in situ allows reliable quantitative analysis. This method, rather lengthy, is sufficiently sensitive. Compared to other methods like mouse or rat test, this method gives a more specific response.

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PROCEDURE FOR THE DETERMINATION OF OKADAIC ACID IN MUSSELS

As ADAM reagent and derivatives are unstable when exposed to light at room temperature, all preparative operations have to been done in a room shielded from day light, with subdued lighting.

1 **Reagents**

- 9-anthraldehyde, 97% Aldrich # 27, 868-8
- hydrazine hydrate, Aldrich # 22, 581-9
- N-chlorosuccinimide 98%, Aldrich # 10, 968-1
- 6,9 mM N-chloorsuccinimide, dissolve 0,09 g in 100 methyl acetaat
- quiniclidine 97%, Aldrich # 19, 760-2
- 6,9 mM quinuclidine, dissolve 0,77 g in 100 ml ethylacetaat
- deoxycholic acid reference standard, Aldrich # 10, 730-1
     stock solution: 10 μg/ml in methanol, stored at 4°C, stable for one year.
     working solutions: from 10 to 0.5 μg/ml by pipetting and dilution of stock solution in a 4 ml glass-vial.
- 9-anthralhydrazon (see 3 below).
- 6,9 mM 9-anthralhydrazon (= 9-anthraldehyde hydrazon) dissolve 0,15 g in 100 ml ethylacetaat
- okadaic acid standard: Promochem, X132 (C.N. Schmidt, Amsterdam), 100 μg/ml in dimethyl formamide.
     stock solution: 10 μg/ml in methanol obtained by dilution of the standard solution, stored at 4°C in the dark, stable for one year.
     working solutions: from 10 to 0.5 μg/ml in methanol by diluting the stock solution.

- solvents (Merck): chloroform, absolute ethanol, hexane, methanol were analytical grade; ethyl acetate was distilled; acetonitrile was chromatography grade. All solvent mixture were freshly prepared before each use.

2 **Apparatus and operating conditions**

- Sorvall Omni-mixer, Dupont Intrument, with 50 ml metal baker.
- Seppak silica cartridge, Waters # 51900.
     The columns were placed in a dessicator after the original packaging was opened, to prevent water absorption.
- Seppak C-18 cartridge, Waters # 51910.

- Heating module, Reacti-Therm, Pierce :
     metallic thermostatic block for test tubes, with nitrogen flowing into each tube.

HPLC system:

Column : two cartridges of 100x30 mm id. glass columns
packed with RP Chromspher C-18, 5 μm particle size
(Chrompack # 28267) with a 10 mm guard column.

Injection: Water Intelligent Sample Processor (WISP),
710B automatic, injection of 10 μl from the methanolic
solutions.

Pump: Waters m45
operating conditions: 0.5 ml/min of acetonitrile/water
90/10 (v/v).

Detection: fluorescence detector Hitachi-Merck F-1020
operating conditions: excitation wavelength at 365 nm,
emission at 410 nm. Sensitivity at 1.0.

Recorder: Kipp & Zonen BD40 with double markers pens
operating conditions: paper speed: 5 mm/min, sensitivity
at 10/20 mV.

3 Synthesis of 9-anthraldehyde hydrazone according to Nakaya et al. (1967)

Crystals of 9-anthraldehyde, 8.8 g in total were dissolved in
150 ml absolute ethanol, in a 250 ml erlenmeyer. The hydra-
zine hydrate (9 ml) was added and the solution was stirred for
three hours at room temperature. The solid product was filte-
red off under low pressure (Whatman nº 5 filter) and dried
under vacuum. The solid was purified by recristallisation from
ethanol, filtered off and washed with the mother solution and
finally dried in an oven at 100°C. The crystals have a MP of
124-126°C (Nakaya et al., 1967).

The light yellow crystals were stored at -18°C.

4 Synthesis of 9-anthryldiazomethane (ADAM), according Yoshida et al. (1988).

For each derivatisation, a new batch of ADAM reagent was
prepared. In a 4 ml-glass vial (WISP vial) with screwed cap,
500 μl of the following solutions in ethyl acetate were mixed:
N-chlorosuccinimide 6.9 mM, 9-anthraldehyde hydrazone 6.9 mM
and quinuclidine 69 mM. The resulting mixture was allowed to
stand at room temperature in the dark. After 30 min of reacti-
on time, this solution was used for derivatisation.

The ethyl acetate solutions of these three components were
stored in the dark, at room temperature, and used within two
months.

5 Derivatisation of standard with ADAM

The derivatisation with ADAM was carried out in a glass micro-tube (300 μl vial insert for WISP vials). 100μl of the ADAM synthesis mixture was added to 100μl or 200μl of the deoxycholic and OA acid solution. The solution was vortex-mixed. The vial was placed in heating-block and was allowed to stand one night at 50°C.

6 Samples preparation

The blank samples were digestive glands (hepatopancreas) of mussels bought on the market and supposed to be free of toxins. The dark brown glands were taken out from fresh or cooked mussels, weighed and stored frozen at -4°C.
For cooked material, the mussels were allowed to cook for 7 to 10 minutes in water. After cooling, the digestive glands were removed from the meat.

For dried material, the entire fresh or cooked glands were lyophylised, and reduced to powder, which was stored in the dark at room temperature.

7 Extraction

For extraction, 2 g of wet sample or 0.5 g of dried sample was used. In a metal baker adapted to the Omni-mixer, 8 ml of methanol 80% (v/v) was added, and homogenised for 5 minutes. The homogenate was centrifuged (3500 rpm) for 10 minutes. An 2.5 ml aliquot of the supernatant was transferred to a test tube, and washed 2 times with 4 ml hexane.

(For experiments on sample extraction with adjusted pH, 3 ml of natrium bicarbonate 0.75% w/v was added to 6 ml of the methanolic supernatant, in order to obtain a pH of 8.)

For the C-18 clean-up, 0.625 ml of the methanolic extract was added to 0.375 ml water (Milli Q), in order to obtain a methanol/water ratio of 50/50 (v/v). An aliquot of 0.5 ml was transferred to the C-18 cartridge column which was first conditionned with 5 ml methanol, and then with 5 ml methanol 60% (v/v). After loading the sample, the column is then eluted with 3 ml of methanol 60% (v/v). The total eluate is extracted with chloroform like described further.

(For the samples without C-18 clean-up, the methanolic extract was added to water in order to have a methanol/water ratio of 60/40. The resulting mixture was then extracted as follows.)

A 3 ml aliquot of the methanolic solution was extracted two times with 4 ml chloroform. The combined chloroform extracts was evaporated at 50°C under nitrogen flow. The residue was dissolved in 300 μl methanol, containing the internal standard DCA (0.1 or 0.05 μg/100 μl).

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8 Derivatisation of extracts with ADAM

The derivatisation with ADAM was carried out in a glass micro-tube (300 μl vial insert for WISP vials). 100μl of the ADAM synthesis mixture was added to 100μl of the extract solution. The solution was mixed, and the vial is placed in thermo-block and allowed to stand one night at 50°C.

9 Derivative clean-up

An aliquot of 100 μl of the derivatised mixture was pipetted in a pointed glass tube, and the solvent was evaporated to dryness under nitrogen at 50°C.

The silica cartridge column was conditionned with 5 ml hexane-/chloroform 50:50 (v/v). The derivatisation residue was trans-ferred with 3 times 1 ml of hexane/chloroform 50:50 (v/v), to the cartridge, and the eluate was discarded. The column was washed with 5 ml of the same solvent, and then with 4 ml chloroform. The derivatives were eluted with 5 ml chloroform-/methanol 95:5 (v/v), and collected in a pointed test tube. After evaporating the solvent to dryness under nitrogen at 50°C, the residue was dissolved in 200 μl methanol, and trans-ferred in 300 μl glass vial, tightly stoppered for storage in darkness until 10 μl were injected into the HPLC system.

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