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On-line determination of carboxylic acids, aldehydes and ketones by high-performance liquid chromatography-diode array detection-atmospheric pressure chemical ionisation mass spectrometry after derivatization with 2-nitrophenylhydrazine

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Abstract

2-Nitrophenylhydrazine (2-NPH) is widely used for the derivatization of carboxylic acids, aldehydes and ketones, in industrial and biological samples. These compounds react with 2-NPH to form derivatives, which are separated by high-performance liquid chromatography (HPLC) and detected with diode array detection (DAD). The UV spectra give information about the functionality of the compounds: carboxylic acid or ketone/aldehyde. Most of the eluting compounds in "known" samples are well characterised by the retention time (comparison with those of standards) of the 2-NPH derivative and their UV spectrum. The identification of different unknown 2-NPH derivatives of carboxylic acids, ketones and/or aldehydes, in industrial or biological samples, based on retention time and/or UV spectrum is not sufficient. These unknown 2-NPH compounds can be identified with on-line atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) based on the molecular mass or/and the fragmentation of the derivative. A novel and specific on-line HPLC–DAD–APCI(−)-MS method is described for the determination of carboxylic acids, ketones and aldehydes, after on-line pre-column derivatization with 2-NHP. The fragmentation of different 2-NPH derivatives were investigated and the possibilities of APCI(−)-MS detection were demonstrated by the on-line identification of an unknown derivative, which turned out to be a side product between 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 2-NPH in the presence of high concentrations of a cyclic amide in the sample solution.

Keywords: Derivatization, LC; Detection, LC; Carboxylic acids; Carboxyl compounds; Nitrophenylhydrazine

1. Introduction

The quantification and identification of a variety of aliphatic carboxylic acids and aldehydes/ketones at sub ppm levels in an aqueous environment is very important in the field of chemical, polymer and life sciences, e.g. chemistry with water as the main solvent is very attractive. Another example is the study of migration of small amounts of carboxylic acids, ketones and/or aldehydes from plastic barriers into bottled water. These compounds are of major importance for taste and thus, the final water quality [1]. Obviously, low detection limits of these carboxylic acids, aldehydes and ketones are necessary. However, most aliphatic carboxylic acids, aldehydes and ketones show not enough absorption with UV-Vis detection.

Several high-performance liquid chromatography (HPLC) methods, with a variety of detection methods, have been developed for the analysis of aliphatic carboxylic acids, aldehydes and ketones, including various pre-column derivatization techniques (UV and fluorescence labelling) to perform selective separation and sensitive detection/analysis of acids, aldehydes and ketones [2]. About a decade ago we compared four different HPLC methods for the determination of trace amounts of polar aliphatic mono-carboxylic acids, ion-suppression reversed-phase HPLC with UV detection using on-column concentration (injection volume of 1000 μl aqueous solution) and direct detection at low wavelengths ($\lambda < 210$ nm), high-performance ion-exclusion

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chromatography with suppressed conductimetric detection, a pre-column derivatization method with 4-bromomethyl-6,7-dimethoxycoumarine (BMMC) with fluorescence detection and pre-column derivatization with 2-nitrophenylhydrazine (2-NPH) hydrochloride with UV-Vis detection [3]. The ion-suppression reversed-phase HPLC method with low wavelength detection, is often problematic because organic contaminations in the injected samples and/or in the mobile phase can cause also absorbance in this low-UV region. Large injection volumes of "clean" aqueous samples are necessary to obtain low limits of detection (100 μg/l). Other detection methods such as refractive index (RI) and evaporative light scattering detection (ELSD) will not solve the lack in selectivity. The gradient used for the separation of the carboxylic acids makes RI not suitable, while ELSD has no or too poor sensitivity because of the high volatility of the small carboxylic acids. Direct HPLC separation combined with sensitive and selective mass spectrometric (MS) detection (LC MS and LC MS–MS) is becoming very popular for carboxylic acid analysis to increase the sensitivity and selectivity, especially in the field of biochemistry. Electrospray ionisation (ESI) in the negative mode is highly sensitive towards carboxylic acids, which makes LC MS, without complicated sample treatment of the samples, very useful for the analysis of acids [4]. Detection limits of 2.3 pg for eicosapentaenoic acid (fatty acid) in seafood and other biological samples and linear calibration curve up to 0.1 μg/l using LC ESI(−)-MS with selected ion monitoring (SIM) have been recently reported [5]. Aldehydes and ketones are substantially less sensitive with MS detection compared to carboxylic acids [4]. To increase the MS sensitivity of aldehydes and ketones pre-column derivatization was performed [6].

The approach to increase sensitivity and selectivity for the analysis of carboxylic acids, aldehydes and ketones, is pre-column derivatization of the carboxylic acids, aldehydes and ketones with specific detection. Most of pre-derivatization methods for carboxylic acids, aldehydes and ketones that are presented in the literature, are based on the reaction with hydrazines [7,8]. The main advantage of derivatization with hydrazines using coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) with respect to other derivatization reagents is the fact that the reaction can be carried out under very mild conditions (weakly acidic medium at 60 °C for 15 min) in aqueous environment [7]. Aldehydes and ketones also react with 2-NPH under similar conditions as carboxylic acids. The fully automated 2-NPH-method reported by Coenen et al. [3] for the determination of polar carboxylic acids is sensitive (detection limit of 50 μg/l), selective (UV-Vis detection at 400 nm) and precise after automatization (4% at 1 ppm level). This makes pre-column derivatization of carboxylic acids, ketones and aldehydes with 2-NPH, followed by RP-HPLC separation and UV detection more preferable, with respect to selectivity and sensitivity, than the described analysis methods without pre-column derivatization.

Fig. 1a shows the reaction mechanism in an aqueous environment between a carboxylic acid and 2-NPH to acid hydrazine (2-NPH derivatives), using EDC as coupling agent. The first step in this reaction is the activation of a carboxylic acid with EDC, catalysed by pyridine [9]. After the activation, two theoretical reaction mechanism (Fig. 1, reactions I and II) are possible to obtain the carboxylic hydrazine derivative [10,11], but reaction I is probably dominant because the formation of an anhydride in aqueous environment is energetically unfavourable. An aldehyde or ketone react with 2-NPH using EDC as a coupling agent by a similar reaction mechanism to another derivative than the carboxylic acid, as shown in Fig. 1b. The 2-NPH derivative of an acid gives absorption maxima wavelengths $\lambda = 230$ and 400 nm at pH < 8, but UV-Vis detection at 400 nm is preferable because the remainders of reagent and reaction by-products have poor absorption at $\lambda = 400$ nm, in spite of the four-fold lower sensitivity [7]. The 2-NPH derivatives of aldehydes and ketones have an absorption maximum at $\lambda = 445$ nm. The UV spectra provide information about the functionality of the compounds: carboxylic acid or ketone/aldehyde. Most of the eluting compounds are characterised by comparison of their retention time with those of standards and their UV spectrum.

The identification of different unknown 2-NPH derivatives of carboxylic acids, ketones and/or aldehydes, in industrial or biological samples, based on retention time and/or UV spectrum is not sufficient. Molecular weight of the eluting compounds can be obtained by MS detection, while the structure of the compounds can be elucidated by means of fragmentation through collision-induced dissociation (CID) in, for example, the transport region of a single quadrupole MS system (also called "pseudo" MS^2) [12] or in an ion-trap MS system [4]. The fragmentation of 2,4-dinitrophenylhydrazine (DNPH) and its propane derivative with HPLC atmospheric pressure chemical ionisation in the negative mole [APCI(−)] multiple mass spectrometry (MS^n) was investigated by Kölliker et al. [13] and can be used for structural elucidation of DNPH derivatives. The electron-affinity of the nitrophenyl-group makes APCI(−)-MS very suitable for identification of unknown 2-NPH derivatives, based on the molecular mass or/and the fragmentation of the derivatives.

A novel and specific on-line method is described, which is based on the determination of carboxylic acids, ketones and aldehydes after on-line pre-column derivatization with 2-NPH, with on-line APCI(−)-MS and UV detection. The MS fragmentation of different 2-NPH derivatives were described and the added value of on-line APCI(−)-MS is demonstrated by the identification of an unknown derivative (artefact of the derivatization reaction when cyclic amides are present) when analysing the concentration of carboxylic acids in aqueous solutions.

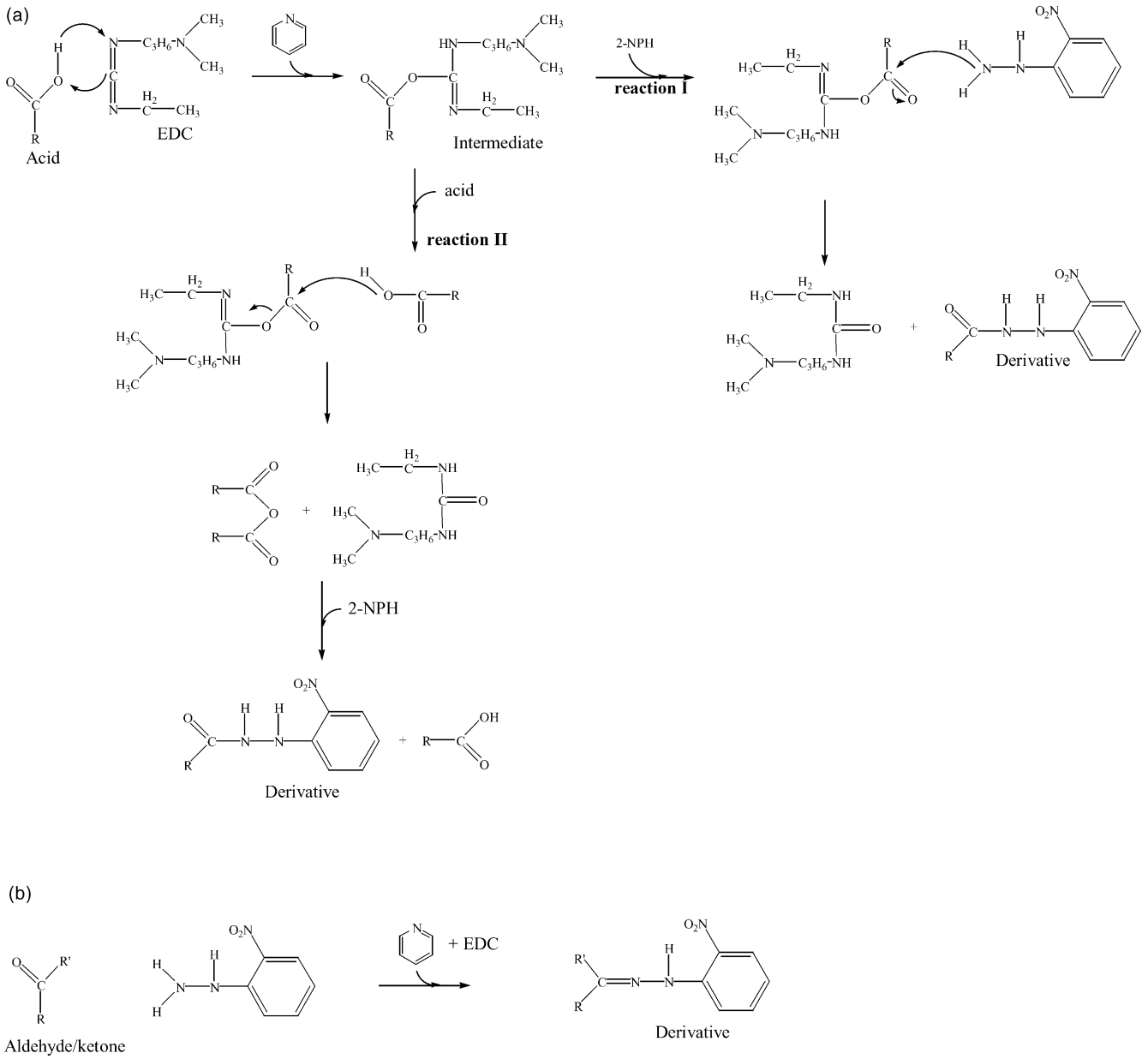

Fig. 1. Schematic reaction of the carboxylic acids and 2-NPH, using EDC as coupling agent, catalysed by pyridine.

2. Experimental

The 2-NPH reagents consisted of 62 mg 2-NPH (97%, Janssen Chimica, Beerse, Belgium) in 5 ml ethanol (pro analysis, Merck, Darmstadt, Germany). After dissolved by ultrasonic agitation (Branson 5210, Danburry, USA) at ambient temperature, 2 ml 1 mol/l HCl (37% HCl, Merck, Darmstadt, Germany) and 3 ml Milli-Q water (Waters, Milford, MA, USA) was added and mixed (0.04 mol/l 2-NPH in 50% aqueous ethanol). The 2-NPH reagent solution was prepared fresh daily. The EDC solution was prepared by dissolving 0.24 g EDC (Sigma, St. Louis, MO, USA) in 5 ml Milli-Q water and was stored for maximum 1 week at 4 °C (0.25 mol/l EDC). The working solution of EDC was prepared fresh daily by mixing an equal part of a 3% solution of pyridine (Merck) in ethanol and an equal part of the EDC solution. All the carboxylic acids, ketones and aldehydes used, was purchased from Fluka (Buchs, Switzerland). To increase the solubility of higher carboxylic acids, ketones and aldehydes, all samples and calibration solutions were diluted with Milli-Q water (Millipore, Milford, MA, USA) and acetonitrile (ACN, analytical-reagent grade, Merck) until the concentration of acetonitrile is 50% (v/v).

All separations, followed by diode array detection (DAD), were performed with a Thermo Separations Products (Thermo Electron Corpo., San Jose, CA, USA) liquid chromatography system, which consisted of a P400 binary pump, an AS3000 autosampler (inclusive vortex mixing unit and heating oven) with column oven and an UV6000LP diode array detector, equipped with an extra long light pipe UV

Table 1
The automatic on-line 2-NPH derivatization and injection protocol

Step	Action
1	Draw 40 μl 2-NPH reagent solution
2	Eject in reaction vial
3	Draw 80 μl EDC working solution
4	Eject in reaction vial
5	Draw 400 μl sample
6	Eject in reaction vial
7	Mix reaction vial for 0.5 min with vortex mixer
8	Heat mixture in reaction vial for 15 min at 60 °C
9	Mix reaction vial for 0.5 min with vortex mixer
10	Inject 100 μl of mixture in reaction vial
11	Start LC analysis

cell ($L = 50$ mm, $V = 10$ μl). Run control and UV-DAD data analysis was performed with ChromQuest, Version 3.0 (ThermoQuest Corporation, San Jose, CA, USA). The separation was performed with a 250 mm × 4 mm Nucleosil 120-5-C_{18} reversed-phase column (Machery Nagel, Düren, Germany). The column temperature was maintained at 40 °C. The analyses were carried out gradiently using mobile phase A, which consisted of acidified water with HCl (pH 4.5) and mobile phase B, which consisted of ACN (analytical-reagent grade Merck). The gradient started at $t = 0$ min with 92% (v/v) A, changed to 80% (v/v) A at $t = 12$ min and reached 60% (v/v) A at $t = 20$ min. A changed at $t = 25$ min to 30% (v/v) A and where it remained constant for 4 min. At $t = 30$ min the eluent reached the final conditions of 10% (v/v) A and 90% (v/v) B. The stoptime was 41 min and the flow rate was 2 ml/min. The pre-column derivatization and the injection protocol were automated using an autosampler with vortex mixing unit and heating oven (see Table 1). The derivatives were detected using DAD at $\lambda = 400$ and 445 nm. The MS experiments were performed using an on-line coupled HP1100 single quadrupole mass spectrometer (Agilent, Waldbronn, Germany) equipped with an APCI interface, with a corona needle shortened in the laboratory to prevented arcing in the interface caused by the high concentration of chloride ions in the mobile phase. ChemStation revision A08.03 software (Agilent) was used for data acquisition and data analysis. The MS system was run in negative mode at an ionisation voltage of 3 kV, corona current of 25 μA, fragmentor voltage between 50 and 200 V, scan range of *m/z* 50–600, step size 0.20 and full data storage. The eluent flow was split (1:3) by means of a zero dead volume T-piece to assure a flow of approximately 0.7 ml/min into the APCI interface. The interface gas and the vaporiser temperature were held constant at 350 °C and the nebulizer pressure was set on 50 psi (1 psi = 6894.76 Pa). Dry nitrogen at a flow rate

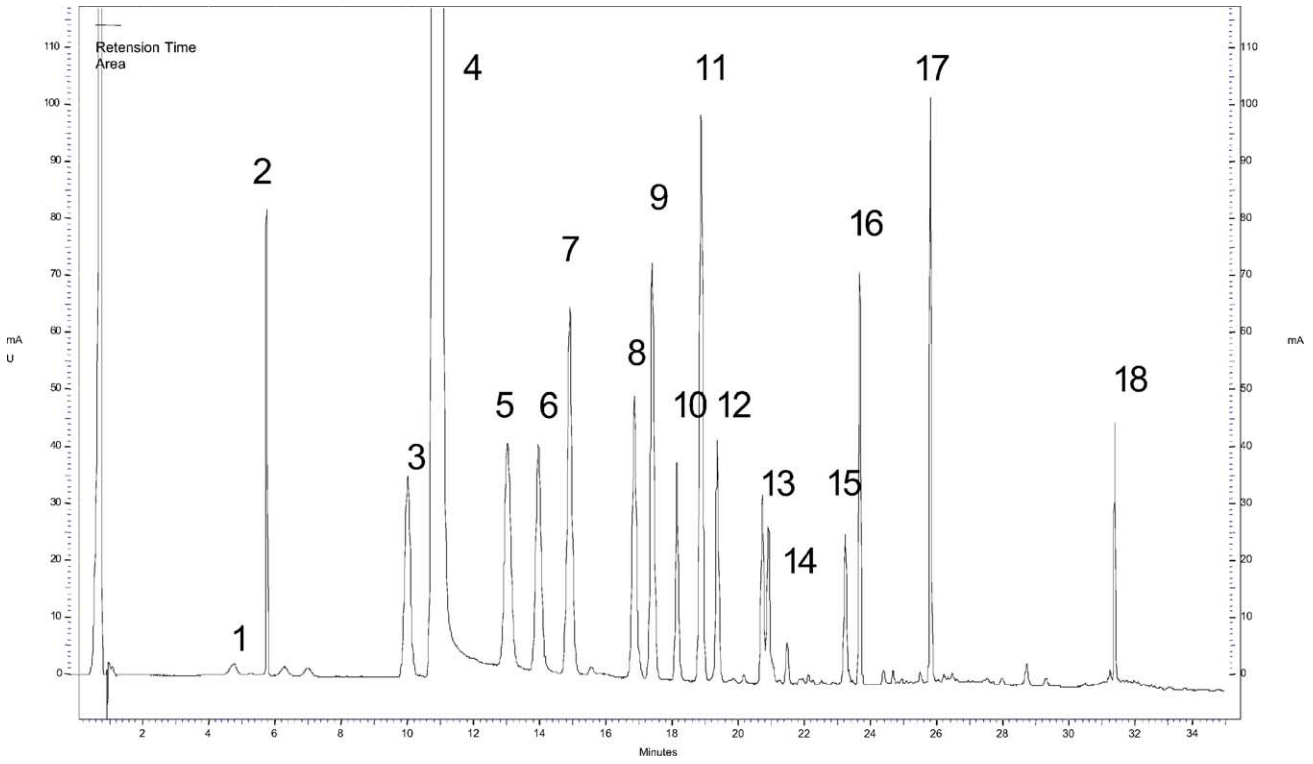

Fig. 2. Chromatogram of a representative synthetic mix of 14 different carboxylic acids at the concentration level of 5 mg/l. Conditions: mobile phase A, acidified water pH 4.5 and B: acetonitrile, flow: 2 ml/min, column: 250 mm × 4 mm Nucleosil 120-5-C_{18} at 40 °C, gradient: $t = 0$ min 92% (v/v) A, $t = 12$ min 80% (v/v) A, $t = 20$ min 60% (v/v) A, $t = 25$ min 30% (v/v) A, $t = 29$ min 30% (v/v) A, $t = 30$ min 10% (v/v) A, pre-column derivatization and injection protocol see Section 2, UV detection at $\lambda = 400$ nm. Peaks: 1, glycolic acid; 2, heptanedioic acid; 3, propenoic acid; 4, NPH; 5, 3-butenoic acid; 6, 2-methylpropanoic acid; 7, butanoic acid; 8, 4-pentenoic acid; 9, 3-pentenoic acid; 10, 2-pentenoic acid; 11, pentanoic acid; 12, benzoic acid; 13, heptanedioic acid; 14, cyclohexenoic acid; 15, blank; 16, heptanoic acid; 17, nonanoic acid; 18, tetradecanoic acid.

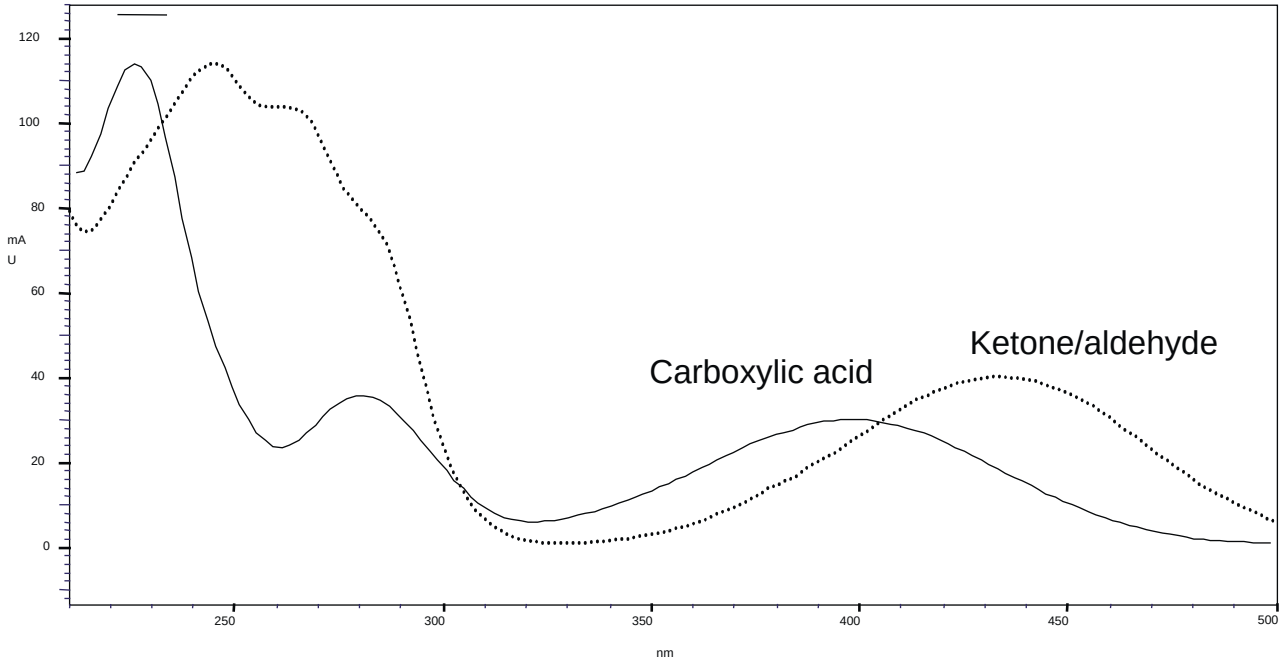

Fig. 3. UV spectrum of a 2-NPH derivative of a carboxylic acid (solid line), respectively the UV spectrum of 2-NPH derivative of a ketone/adehyde (dotted line).

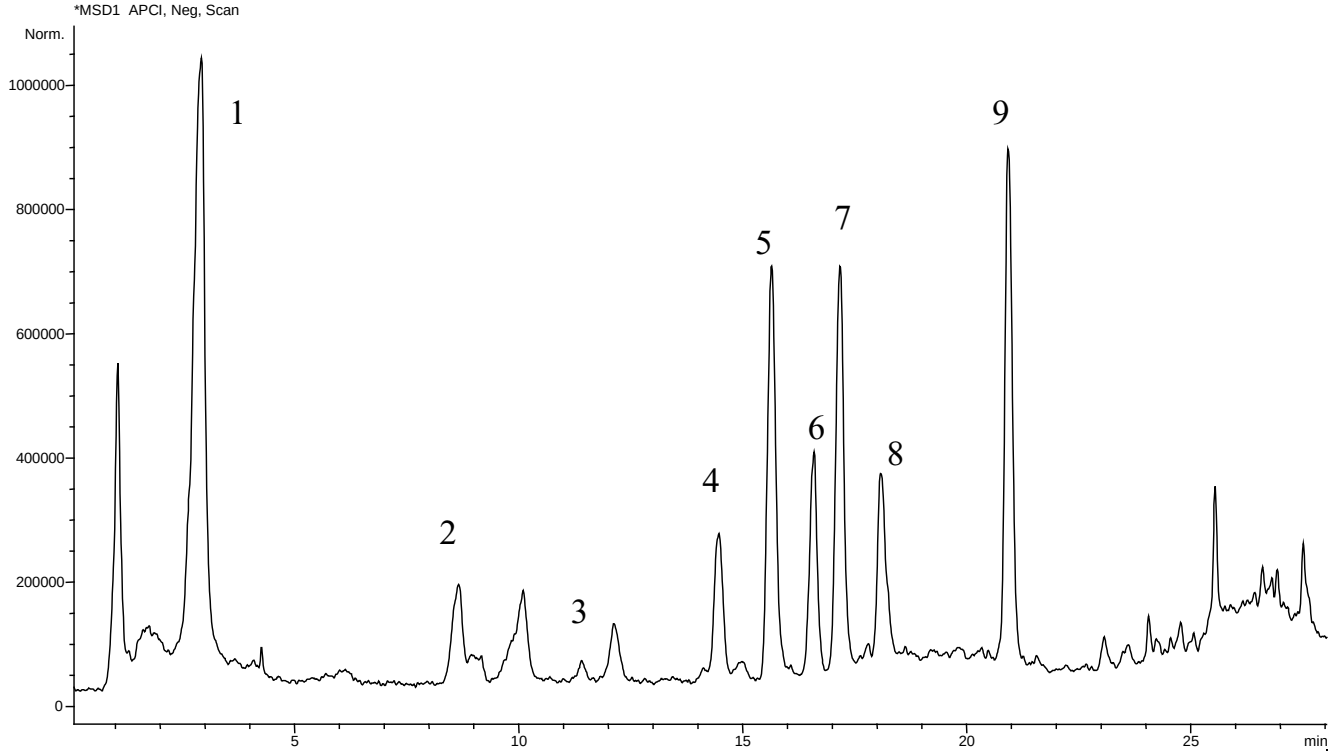

Fig. 4. TIC chromatogram of an industrial sample. Conditions: mobile phase A: acidified water pH 4.5 and B: acetonitrile, flow: 2 ml/min, column: 250 mm × 4 mm Nucleosil 120-5-C_{18} at 40 °C, gradient: $t = 0$ min 92% (v/v) A, $t = 12$ min 80% (v/v) A, $t = 20$ min 60% (v/v) A, $t = 25$ min 30% (v/v) A, $t = 29$ min 30% (v/v) A, $t = 30$ min 10% (v/v) A, pre-column derivatization and injection protocol see experimental section, APCI(−)-MS detection. Peaks: 1, acetic acid; 2, propanoic aicd; 3, butanoic acid; 4, unknown $m/z = 246$; 5, unknown $m/z = 246$; 6, pentanoic acid; 7, cyclopentanecarboxylic acid; 8, 5-hexenoic acid; 9, hexanoic acid.

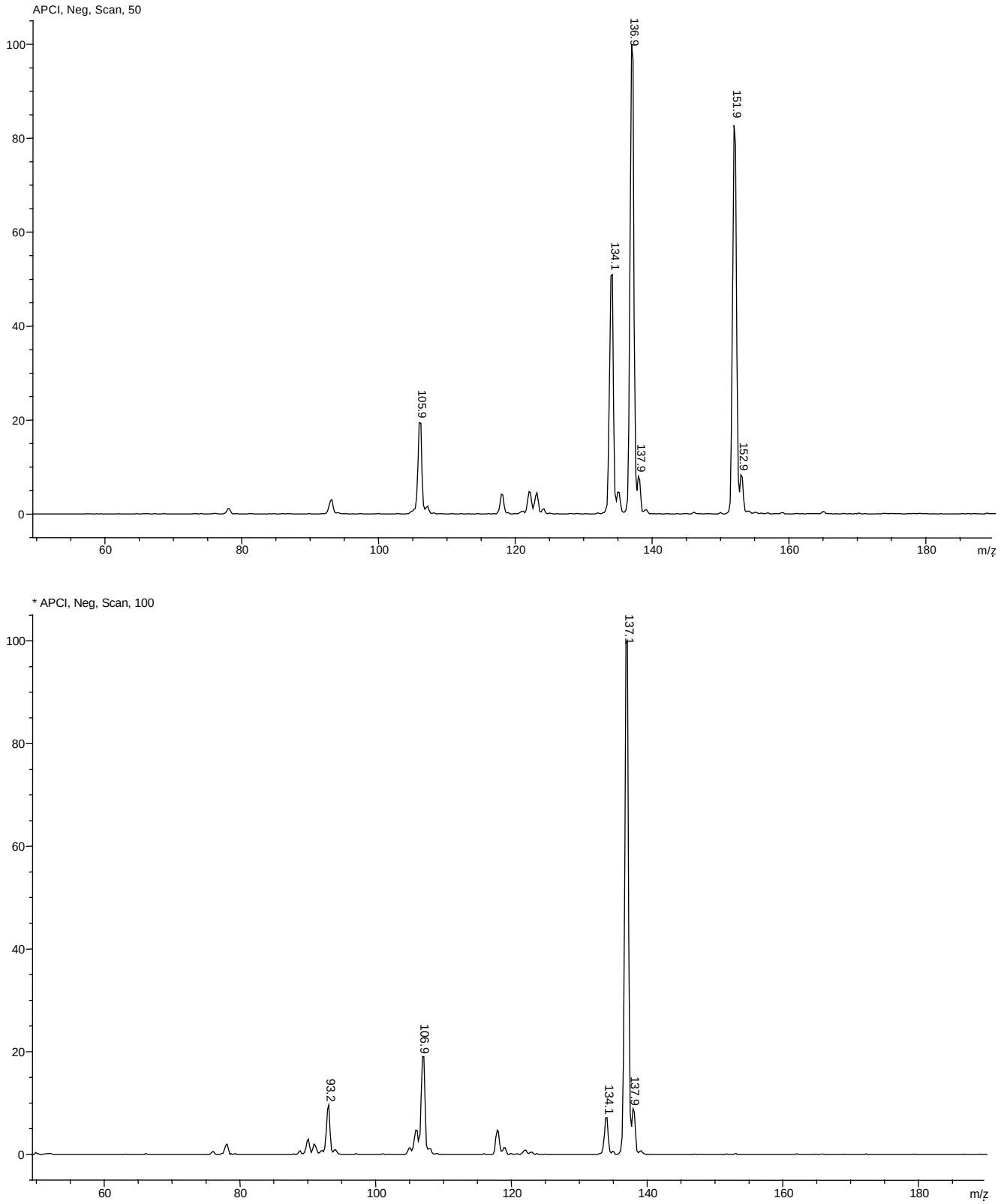

Fig. 5. APCI(−)-MS spectrum of 2-NPH at 50 V fragmentor voltage (up) and 100 fragmentor voltage (down).

of 10.0 ml/min was used as nebulizer gas. An LCQ Deca XP ion-trap MS (Thermo Finnegan, FL, USA) was used to investigate the fragmentation of different compounds. The samples were introduced through the APCI, negative mode, by infusion of the sample solution at 3 μl/min with a syringe pump. The ionisation voltage was −5 kV, discharge current of 4.5 μA, scan range of *m*/*z* 60–800 and full scan mode. The vaporiser temperature was 450 °C, and nitrogen was used as sheath gas. The capillary was held at 200 °C. The spectra were collected in full scan mode, scanning from *m*/*z* 60–1000 in 30 ms. The mass spectral data were processed with Xcalibur Version 1.3 software (Thermo Finnegan). The spectra shown are average spectra form at least 15 scans.

3. Results and discussion

3.1. Separation and quantification with UV

A mixture of carboxylic acids, ketones and aldehydes were pre-column derivatised with 2-NPH, separated with RP-HPLC, and detected with UV absorption. An example of an UV-chromatogram at λ = 400 nm of a representative synthetic mix of 14 different carboxylic acids at the concentration level of 5 mg/l is shown in Fig. 2. All acids give a 2-NPH derivative, which is retained under the stated LC conditions. Only the di-carboxylic acid, heptanedioic acid ($HOOCC_5H_{10}COOH$), gave two peaks at 6.4 and 20.9 min, which were caused by the mono- and di-derivative of 2-NPH. The same phenomena was observed for other di-carboxylic acids like ethanedioic, ethenedioic, propanedioic, butanedioic acids, but also phthalic acids gives the mono- and di-derivatives of 2-NPH. For oxalic acid ($C_2H_2O_4$), no derivative with 2-NPH was observed. This indicates that the used reaction conditions are probably not optimal for all the different carboxylic acids. Miwa and Yamamoto [14,15] used a second derivatization step at high pH to obtain only mono-derivatives. In our lab we were not successful at selectively removing the di-derivatives in this way.

Molar response factors of the different carboxylic acids were determined by injecting eight different concentrations in the range of 0.1–10 mg/l of a mixture of different acids after on-line 2-NPH derivatization and analysing as described

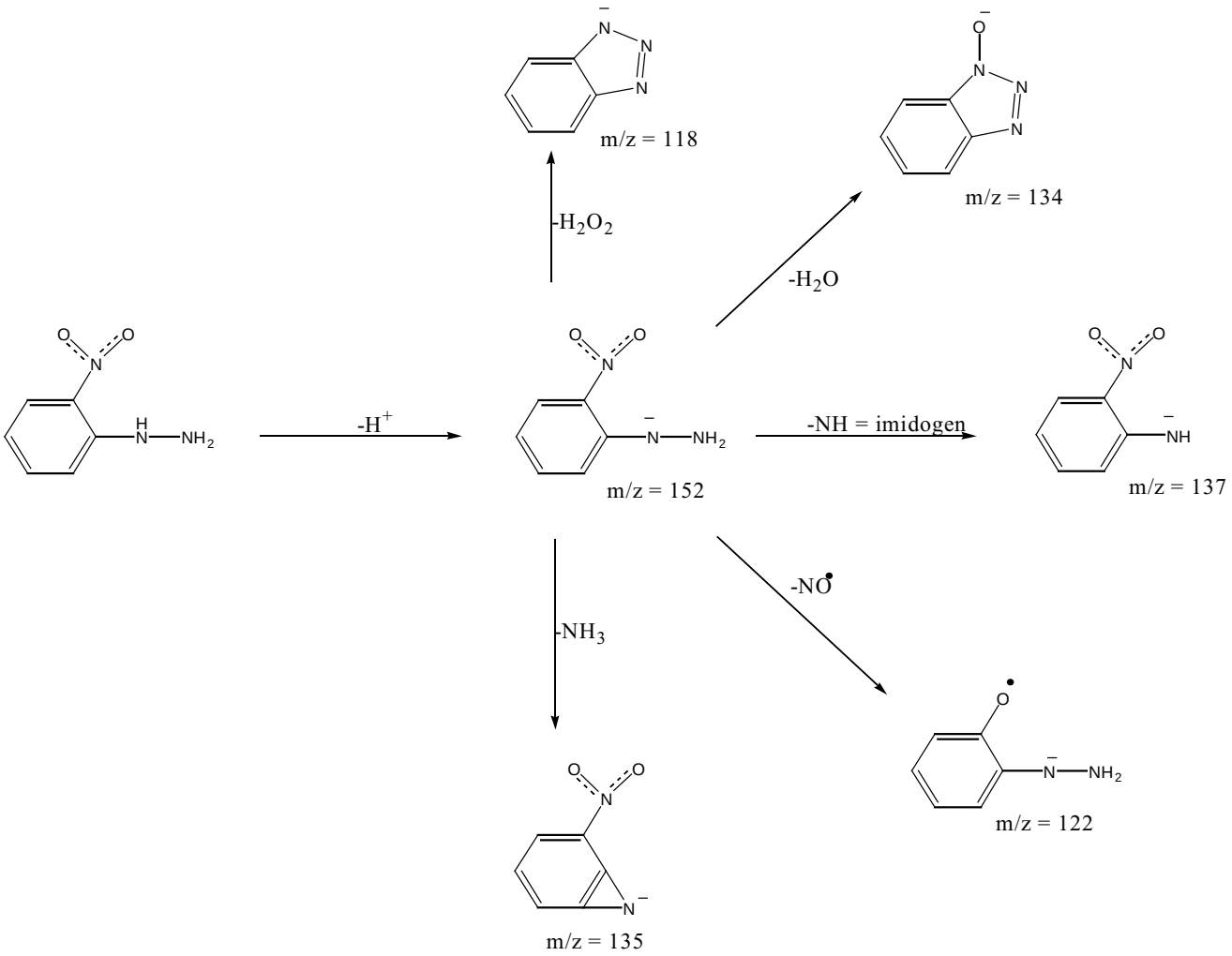

Fig. 6. Proposed fragmentation of 2-NPH.

in the Section 2. Table 2 lists the molar response factors for the different linear carboxylic acids, which are constant (R.S.D. $< 4\%$, $n = 8$) for each carboxylic acid in the measured range of 0.1–10 mg/l. The limit of detection is 50 μg/l (80 pmol of pentanoic acid derivative injected), based on a signal-to-noise ratio of three. These data are in agreement with previous work [3,7]. The UV absorption at $\lambda = 400$ nm is mainly caused by the aromatic part of the 2-NPH derivative, which theoretically should result in an equivalent molar response factor for the different carboxylic acids. The measured molar response factors are steadily decreasing when the alkyl chain of the carboxylic acid is increased. Optimum reaction conditions are likely to differ with different carboxylic acids, so the applied conditions are probably not optimal for all the different carboxylic acids and could cause the non-equivalent molar response factor. The relation between the number of C-atoms and the molar response factor can be fitted with a polynomial equation ($y = 78x^2 - 1838x + 24841$, $R^2 = 0.9213$, $x =$ number of C-atoms, $y =$ molar response factor). Thus, a universal molar response function can be applied to perform quantitative estimation of the concentration of a carboxylic acid, aldehyde and ketone. More accurate results can be obtained if the specific compound is injected as an external standard; to improve the precision internal standardisation should be utilised.

Table 2
Average molar response factors for different linear carboxylic acids (R.S.D. $< 4\%$, $n = 8$) determined with LC DAD ($\lambda = 400$ nm)

Carboxylic acid	Molecular mass 2-NPH derivative (g/mol)	Molar response factor (area/μmol/l)
Acetic acid	195	22230
Propanoic acid	209	19646
Butanoic acid	223	18955
Pentanoic acid	237	16827
Hexanoic acid	251	16064
Heptanoic acid	265	15635
Octanoic acid	279	15624
Dodecanoic acid	307	15350
Tetradecanoic acid	321	13482

3.2. Identification by APCI(−)-MS

The eluted compounds can be identified by comparing their retention times with the retention times of standards.

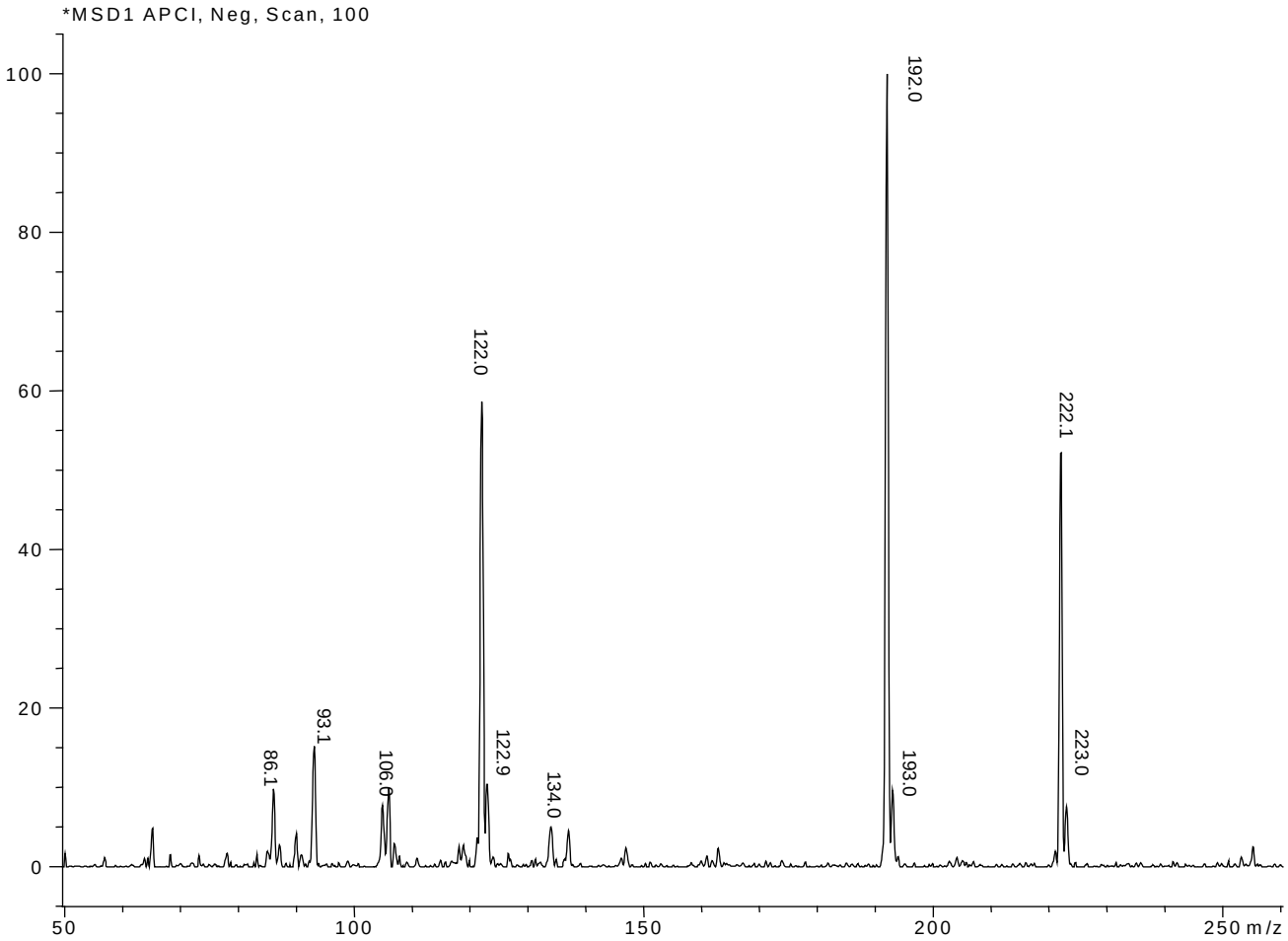

Fig. 7. APCI(−)-MS spectrum of 2-NPH derivative of butanoic acid at high fragmentor voltage (100 V).

The UV spectrum can be used to discriminate between a carboxylic acid and a ketone/aldehyde derivative. The derivative of a carboxylic acid has an absorption maximum at $\lambda = 400$ nm, while the derivative of a ketone or aldehyde has an adsorption maximum at $\lambda = 445$ nm (see Fig. 3). The identification of different unknown 2-NPH derivatives of carboxylic acids, ketones and/or aldehydes, in industrial or biological samples, based on retention time and/or UV spectrum is not sufficient. These unknown compounds can be identified with the help of on-line MS. Identification is based on the molecular weight or/and the fragmentation of the derivative. Different ionisation interfaces (ESI and APCI) and modes (negative and positive ionisation) were investigated. Negative ionisation with APCI gave almost no loss of sensitivity compared to the ESI (positive and negative) and APCI (positive), APCI(−) gave also the highest selectivity as 2-NPH derivatives are ionised and detected. The experimental conditions used for APCI were optimised (see also Section 2). Fig. 4 shows an example of a total ion current (TIC) chromatogram of an industrial sample which contains different derivatised carboxylic acids, which were separated with RP-HPLC and detected with DAD and APCI(−)-MS. The signal-to-noise ratios of the 2-NPH derivatives observed with both APCI(−)-MS and UV detection at $\lambda = 400$ nm are at the same order of magnitude. The apparent retention-time observed with the MS is lower (0.6 min) compared to the UV chromatogram because of a start delay in the MS. The MS data contain information about the molecular weight of the derivatised compound. An unknown compound can be identified by combining the information of the UV spectrum (carboxylic acid or ketone/aldehyde) and the molecular weight. For example, the unknown derivatised compound at $t_R = 15.8$ min (Fig. 4) has an absorption maximum at $\lambda = 400$ nm (a carboxylic acid), with a molecular mass of 112 ($[M-H]^- = 246\ m/z \rightarrow M_{acid} = M_{derivative} \rightarrow M_{2\text{-NPH}} + M_{H_2O} = 247 - 153 + 18 = 112$). From this information the molecular formula of the eluting compound can be extracted: C_5H_7COOH, so the eluting acid is a C_5 alkenoic acid (e.g. 1-cyclopentene-1-carboxylic acid, 2,4-hexadienoic acid).

3.3. Fragmentation of 2-NPH

Structural information of the unknown 2-NPH derivatives can be obtained by fragmentation of the derivative with on-line APCI(−)-MS–MS. For structural elucidation of 2-NPH derivatives it is important to understand the fragment structure and formation. Both the single quadrupole MS and ion-trap MS are used to study the fragmentation of 2-NPH and their different derivatives. First the fragmentation of (unreacted) 2-NPH ($M_r = 153$) was studied by using single quadrupole MS with different fragmentor voltages, see Fig. 5. Most fragments are listed in Table 3. The MS spectrum gives both odd and even *m/z*, which can be caused by the appearance of different amounts of N-atoms or the

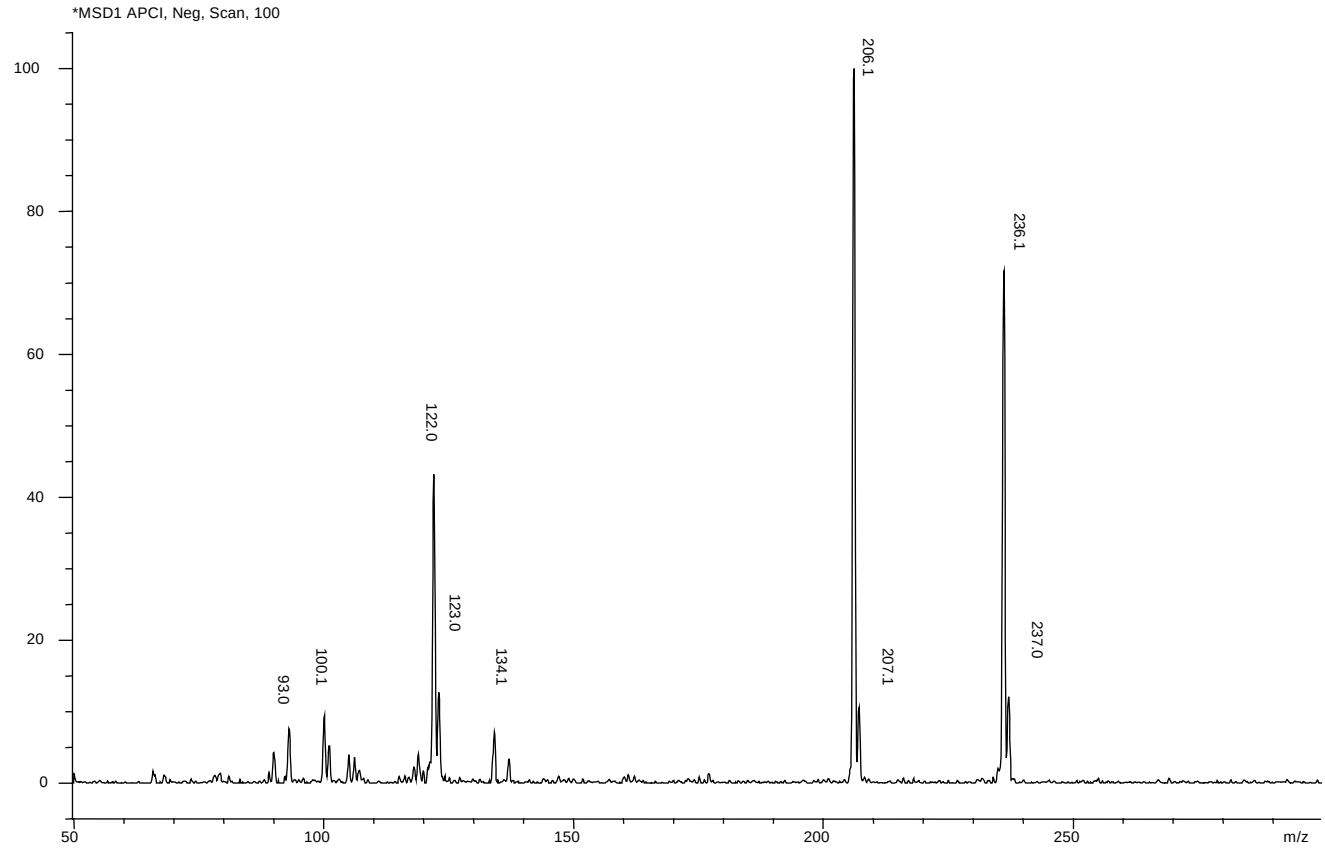

Fig. 8. APCI(−)-MS spectrum of 2-NPH derivative of pentanoic acid at high fragmentor voltage (100 V).

appearance of deprotonated (proton transfer ionisation) and radical anions (electron attachment). The observed loss of 18 (*m/z* 134) was published in detail, while $m/z = 122$ is formed by the loss of a NO radical (30 u) from $m/z = 152$ $[M-H]^-$. The high energies, necessary for the formation of a radical anion ($[M-H-NO]^{-\bullet}$) from an anion, are delivered by the corona discharge. The formation of $m/z = 106$ $[M-H-46]^-$ ion could be explained by the loss of NO_2 or loss of NO radical after an ion–molecule reaction in de ion-trap as proposed by Kölliker et al. [13]. The formation of *m/z* 123 can be explained by the loss of NO radical from the radical anion (*m/z* 153), but no hint was found for this. *m/z* 137 $[M-H-NH]^-$ is a stable ion, which can be explained by the loss of a gas phase molecule imidogen (NH) and the formation of stable delocalised anion. Two main fragments (*m/z* 135 = $[M-H-17]^-$ and *m/z* 122 = $[M-H-30]^{-\bullet}$) are formed from *m/z* 152 $[M-H]^-$ ion of 2-NPH, using the ion-trap MS. The loss of 30 is already described, but the loss of 17 requires a loss of NH_3 to the formation of a three-member ring. The difference in observed fragment ions with the use of the single quadrupole and ion-trap MS can be explained by their difference in how the collisions are performed; fragmentation in the single quadrupole MS is a shorter but higher energy process compared to the fragmentation in the ion-trap MS [4]. The proposed fragmentation of the 2-NPH observed in the MS-spectra is shown in Fig. 6.

Table 3
Main fragments in the MS spectrum of the $[M-H]^-$ ions of 2-NPH at 50, 75 and 100 fragmentor voltage

Mass (*m/z*)	Relative intensity at 50 V	Relative intensity at 75 V	Relative intensity at 100 V	Assignment
152	81	3	0	$[M-H]^-$
137	100	100	100	$[M-H-NH]^-$
134	52	37	6	$[M-H-H_2O]^-$
123	4	2	<1	$[M-H-29]^-$
122	5	4	<1	$[M-H-NO]^{-\bullet}$
118	4	5	6	$[M-H-H_2O_2]^-$
106	18	11	5	$[M-H-46]^-$

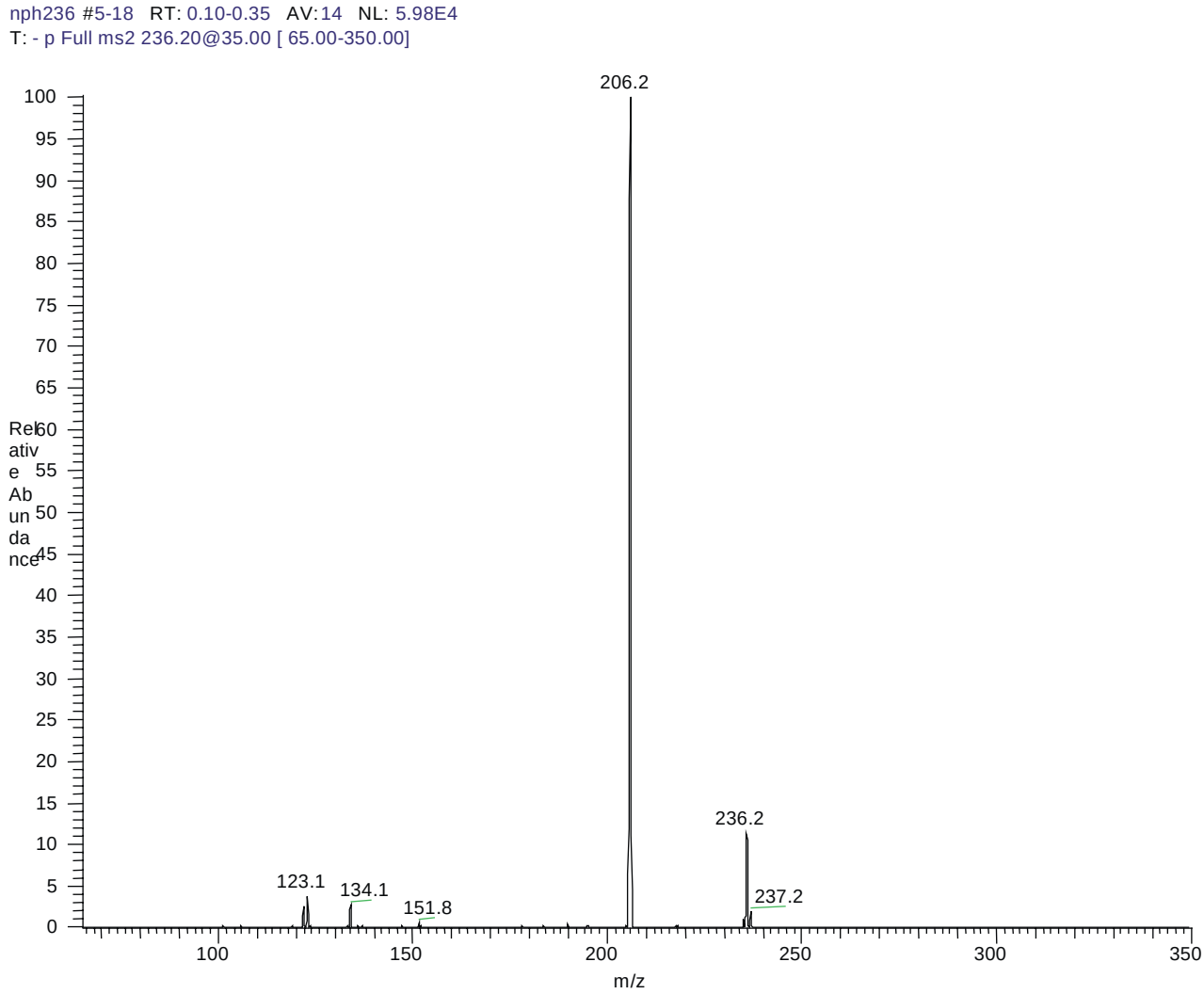

Fig. 9. APCI(−)-MS^2 spectrum of $m/z = 236$ $[M-H]^-$ ion of 2-NPH derivative of pentanoic acid.

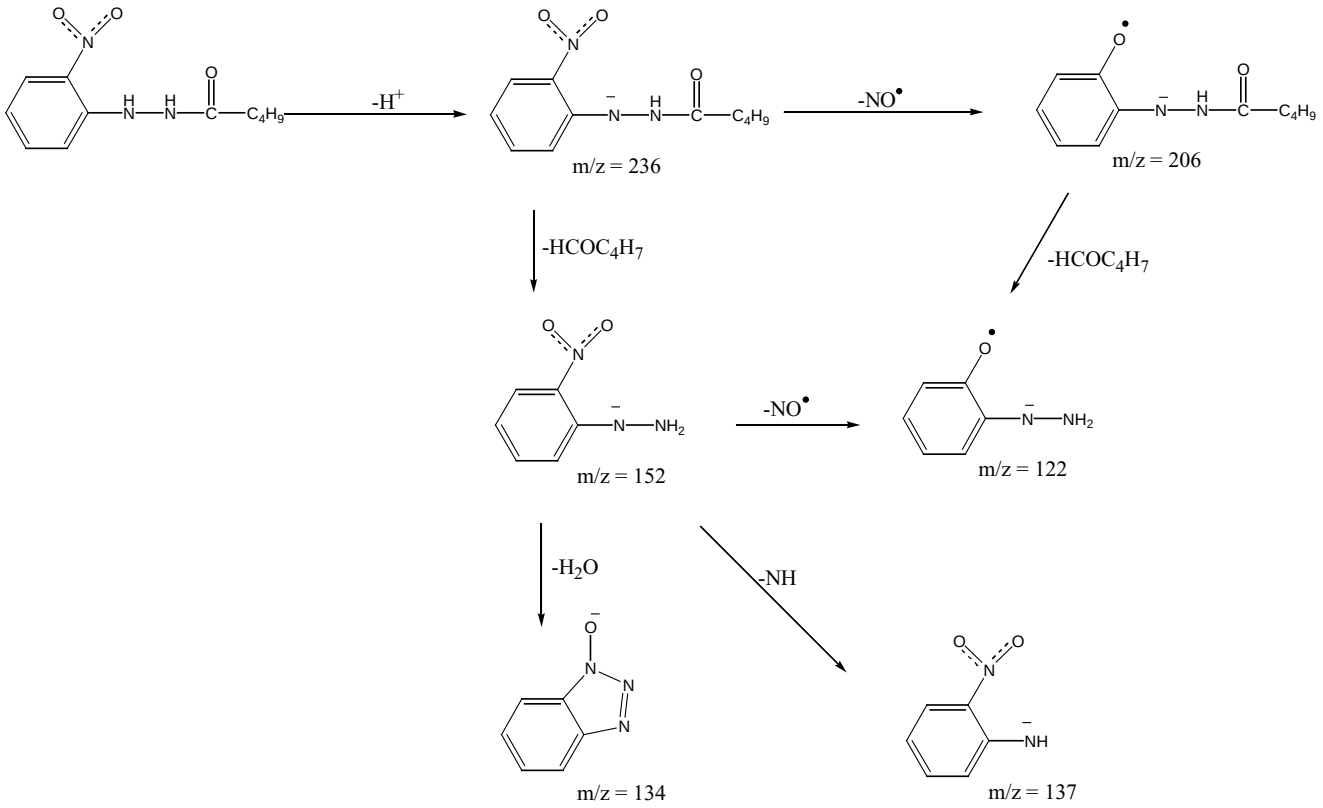

Fig. 10. Proposed fragmentation reaction of 2-NPH derivatives of carboxylic acids.

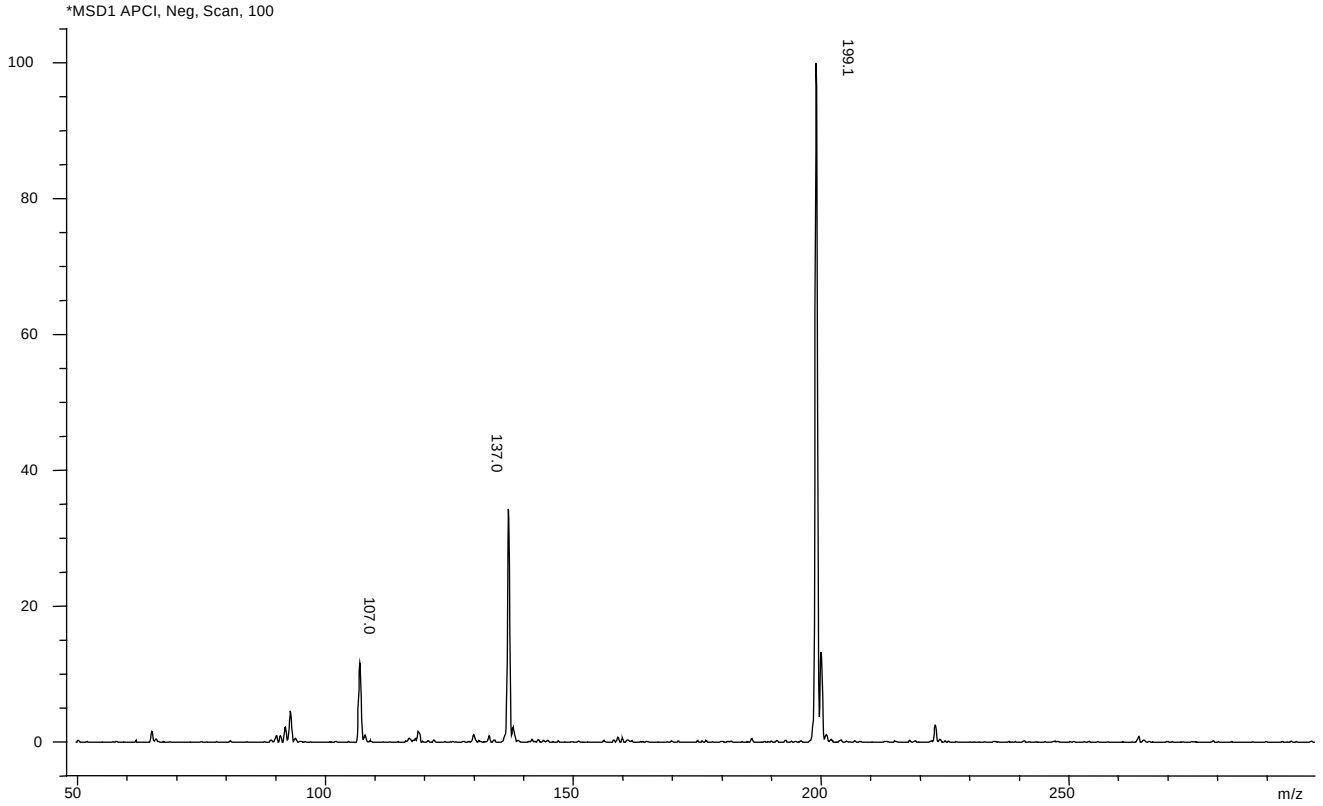

Fig. 11. APCI(−)-MS spectra of the 2-NPH derivative of 2-pentanon at high fragmentor voltage (100 V).

3.4. Fragmentation of carboxylic acids derivatives

The fragmentation of the 2-NPH derivatives of butanoic acid ($HOOCC_3H_7$, $M_r = 88$) and pentanoic acid ($HOOC–C_4H_9$, $M_r = 102$) was investigated using the same "pseudo" MS^2 conditions, see Figs. 7 and 8. The fragments of both derivatives are listed in Table 4. The MS spectra are less complex compared to the MS spectra of 2-NPH. The main fragmentation is a loss of 30 u from $[M-H]^-$ ion, which can be explained by the loss of NO radical from the $[M–H]^-$ ion. Both derivatives show the formation of *m/z* 137 $[m/z = 152–15]^-$, *m/z* 134 $[m/z = 152–18]^-$ and *m/z* 122 $[m/z = 152–30]^{-\bullet}$ ions at higher fragmentor voltages, which are the same fragmentation ions observed in the MS spectrum of 2-NPH. The loss of 30 u was also observed (*m/z* 206) during the fragmentation with the use of the ion-trap MS of $[M-H]^-$ ion of 2-NPH derivative of pentanoic acid (*m/z* 236) and at low intensity the fragment ions *m/z* 152, *m/z* 134 and *m/z* 122 were found (see Fig. 9). The ion intensity of *m/z* 152 in the MS^2 spectrum is too low for MS^3 experiments, while the MS^3 spectrum of fragment $m/z = 206$, show an intense fragment ion of *m/z* 122. The proposed fragmentation reaction is given in Fig. 10.

3.5. Fragmentation of aldehyde and ketone derivatives

The MS spectra of the 2-NPH derivatives of 2-pentanone ($CH_3(C=O)–C_3H_7$, $M_r = 86$) and dodecanal ($O=CH–C_{11}H_{23}$, $M_r = 184$), with "pseudo" MS^2 conditions are shown in Figs. 11 and 12, while the fragments of both derivatives are listed in Table 5. Both derivatives give a strong signal for the radical anion and very stable fragments of *m/z* 199 and 137. The formation of *m/z* 137 can be explained from the fragmentation of 2-NPH, while the formation of *m/z* 107 can be explained by the loss of 30 u (NO radical) from *m/z* 137. The formation of *m/z* 199 would require the impossible loss of 22 u from the radical anion of the 2-NPH derivative of 2-pentanone (*m/z* 221). The formation of *m/z* 199 is investigated using the ion-trap MS (see Fig. 13). The MS^2 spectrum of *m/z* 318 shows the formation of *m/z* 199 and 118 and at low level a loss of 18 (H_2O), 30 (NO radical) and 46 u (NO_2). The MS^3 spectrum of *m/z* 199 gives the formation of *m/z* 123 and a little loss of 18 u (*m/z* 181). It was suspected that this fragment (*m/z* 199) is formed by recombination. This hypothesis can be tested by labelling experiments or high-resolution MS. The proposed fragmentation reaction is given in Fig. 14.

The fragmentation observed and described of the different 2-NPH derivatives can be used for the other unknown 2-NPH derivatives of aldehydes, ketones and carboxylic acids after the RP-HPLC separation.

3.6. Fragmentation of an unknown 2-NPH derivative

An unknown derivative of 2-NPH elutes on the tail of the excess reagents peak when the sample contains

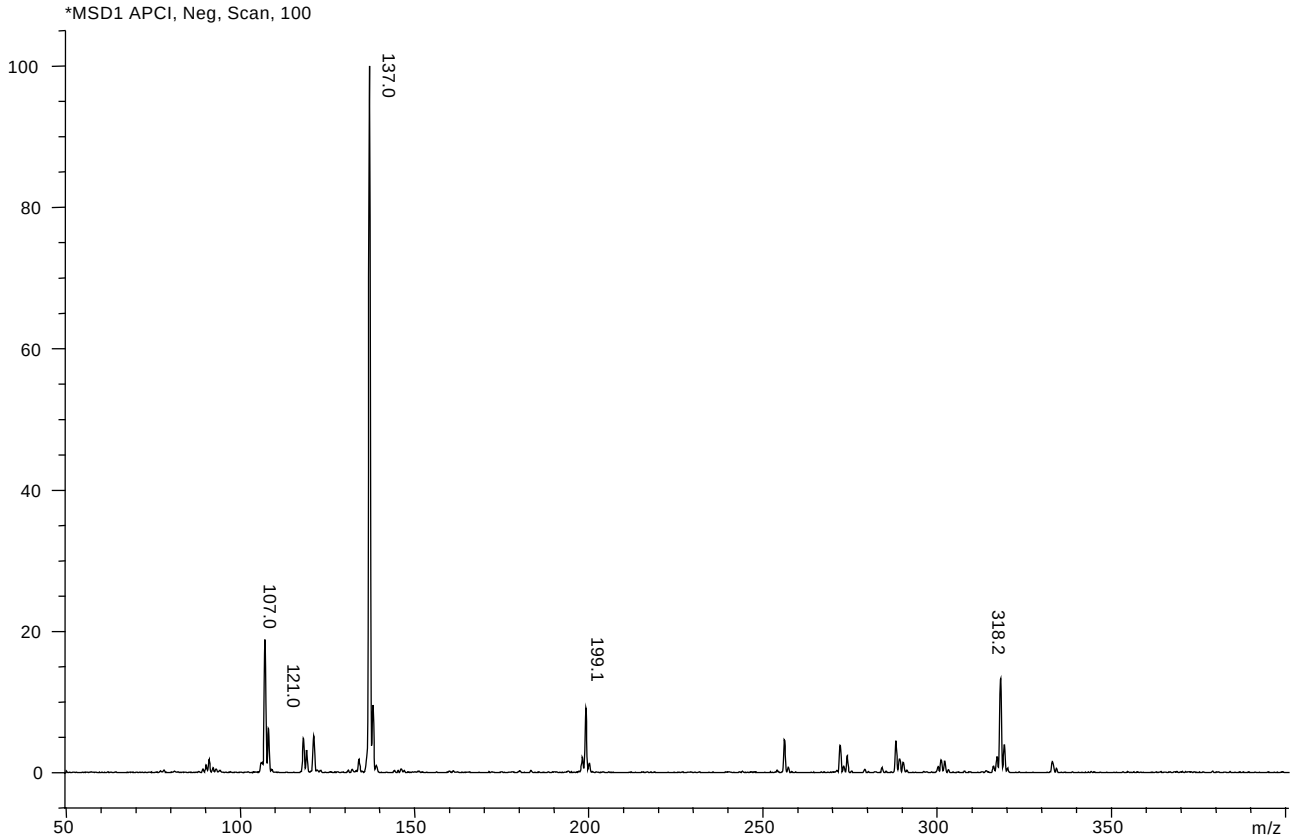

Fig. 12. APCI(−)-MS spectra of the 2-NPH derivative of dodecanal at high fragmentor voltage (100 V).

Table 4
Main fragments in the MS spectrum of the $[M-H]^-$ ion of 2-NPH derivatives of butanoic (*m/z* 222) and pentanoic acid (*m/z* 236) at 50 and 100 fragmentor voltage

Mass (*m/z*)	Relative intensity at 50 V for ions from butanoic acid derivative	Relative intensity at 100 V for ions from butanoic acid derivative	Relative intensity at 50 V for ions from pentanoic acid derivative	Relative intensity at 100 V for ions from pentanoic acid derivative
236	–	–	100	72
222	100	52	–	–
206	–	–	4	100
192	3	100	–	–
137	<2	4	<2	3
134	<2	5	<2	7
122	<2	58	<2	43
118	<2	3	<2	3

a cyclic amide compound at concentrations higher than approximately 0.05% (m/m) (see Fig. 15). The retention time is very reproducible and the UV spectrum looks like the spectrum of a carboxylic acid 2-NPH derivative. Derivatization experiments in the presence of linear amides (e.g. *N*-butylbutyramide, C_3H_7–CO–NH–C_4H_9) and cyclic amides [e.g. 2-oxohexamethylenimine, cyclic-(C_5H_{10}–CO–NH)] show that the unknown derivative only appears in the chromatogram in the presence of cyclic amides, even when no carboxylic acids, ketones or aldehydes are present

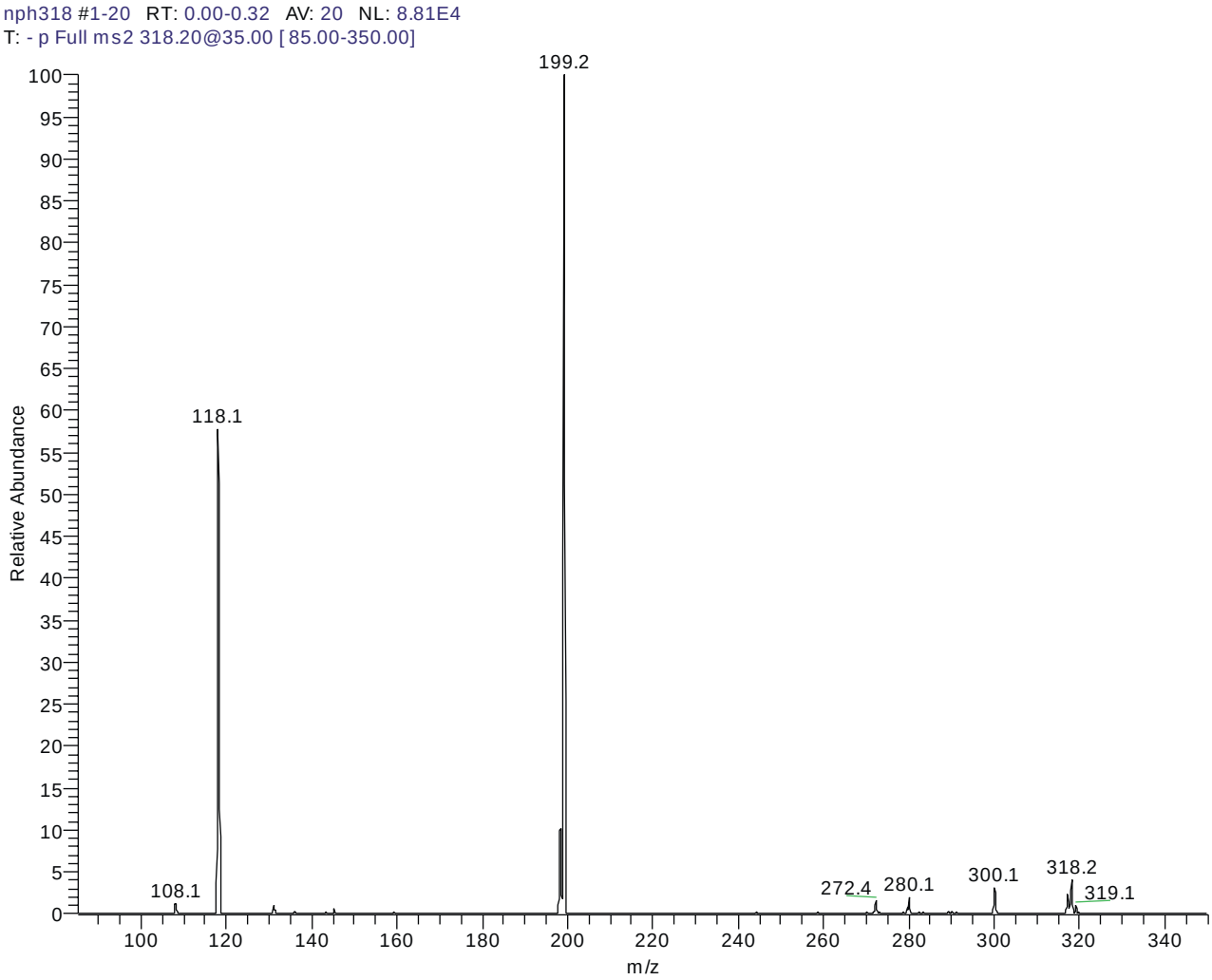

Fig. 13. APCI(−)-MS^2 spectrum of $m/z = 318$ $[M-H]^-$ ion of 2-NPH derivative of dodecanal.

Table 5
Main fragments in the MS spectrum of the $[M-H]^-$ and $[M]^{-\bullet}$ (radical anion) ion of 2-NPH derivatives of dodecanal (*m/z* 318 = $[M-H]^-$) and 2-pentanon (*m/z* 220 = $[M-H]^-$) at 50 and 100 fragmentor voltage

Mass *m/z*	Relative intensity at 50 V for ions from 2-pentanon derivative	Relative intensity at 100 V for ions from 2-pentanon derivative	Relative intensity at 50 V for ions from dodecanal derivative	Relative intensity at 100 V for ions from dodecanol derivative
319 = $[M]^{-\bullet}$	–	–	100	<2
318	–	–	38	14
290	–	–	7	5
221 = $[M]^{-\bullet}$	30	2	–	–
206	<2	<2	–	–
199	100	100	<2	8
137	73	34	40	100
107	<2	12	<2	18

in the analysed solution. The appearance of the unknown peak is independent of the number of C atoms (C_5–C_{12}) of the cyclic amide. It cannot be assumed that this unknown compound is a carboxylic acid, ketone or aldehyde because it is evident that this compound always appeared in solutions that contains a cyclic amide, and was never found in calibration solutions or blanks. It was also observed that the response of this unknown compound is linearly related to the cyclic amide concentration: every 1% (w/w) cyclic amide gives approximately 10 mg/l of the unknown compound using the response factor of butyric acid. It is assumed that this unknown compound is a reaction product from the derivatization reagent 2-NPH. This specific reaction is catalysed by a cyclic amide. Therefore, this compound

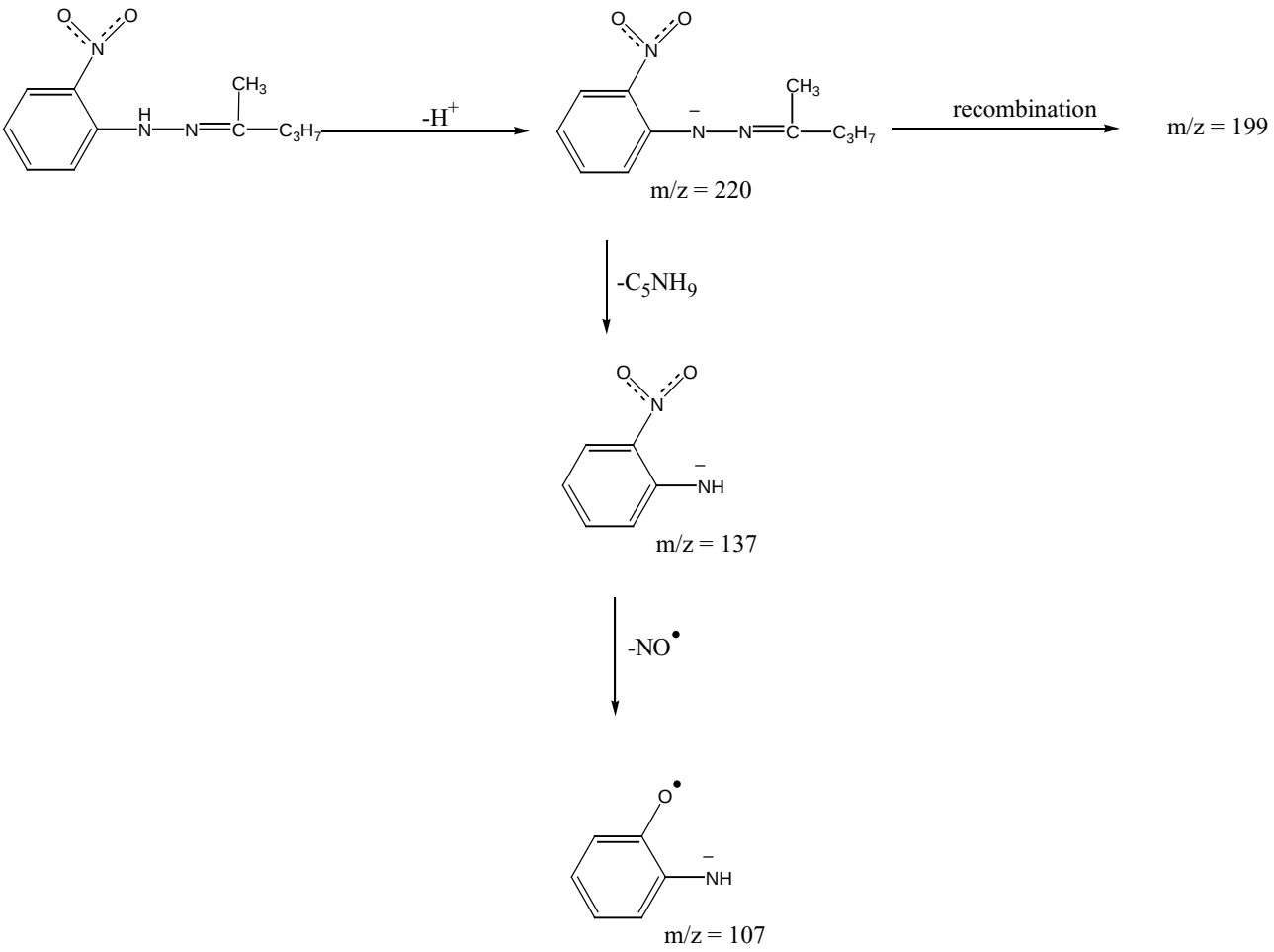

Fig. 14. Proposed fragmentation reaction of 2-NPH derivatives of aldehyde/ketone.

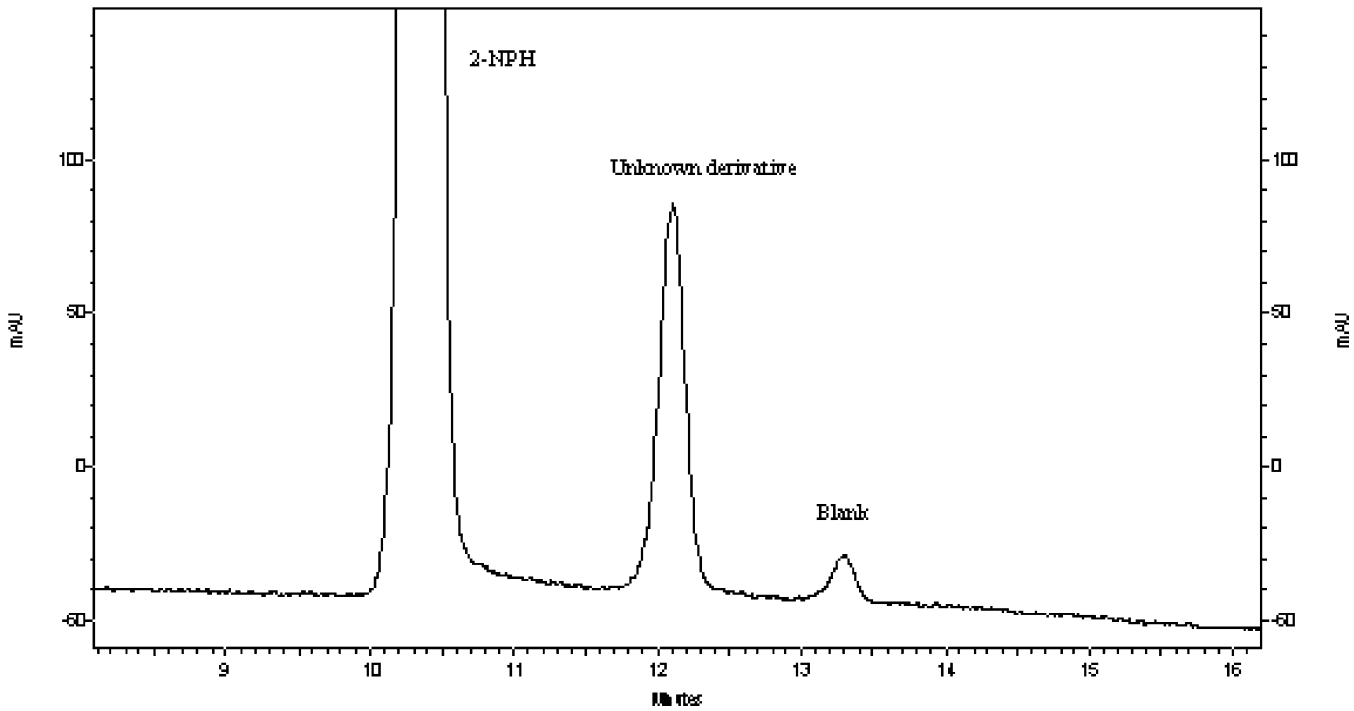

Fig. 15. Chromatogram of unknown 2-NPH derivative. Conditions: mobile phase A, acidified water pH 4.5 and B, acetonitrile, flow: 2 ml/min, column: 250 mm × 4 mm Nucleosil 120-5-C_{18} at 40 °C, gradient: $t = 0$ min 92% (v/v) A, $t = 12$ min 80% (v/v) A, $t = 20$ min 60%(v/v)A, $t = 25$ min 30% (v/v) A, $t = 29$ min 30% (v/v) A, $t = 30$ min 10% (v/v) A, pre-column derivatization and injection protocol see Section 2, UV detection at 400 nm.

is not present in the original sample. On-line APCI(−)-MS experiments, with different fragmentor voltage (50–200 V), were performed to identify this unknown compound. These experiments showed a m/z of 223 ($[M-H]^-$)(see Fig. 16), which means that the molecular mass of the compound before derivatization is 89. The odd molecular mass means that the compound must have an odd number of N-atoms in the structure. The structure of the unknown derivative is identified by verifying the fragmentation pattern of the unknown derivative, see Fig. 17. This compound is probably formed by the reaction between EDC and 2-NPH, while the cyclic amide acts like a catalyst, as shown in Fig. 18. It is

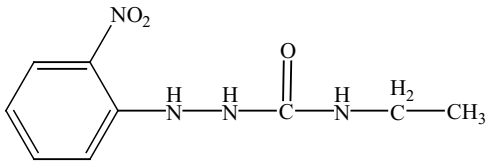

Fig. 17. Structure of unknown derivative.

remarkable that linear amides do not show this catalytic behaviour, which can be explained by the better steric accessibility of a cyclic amide compared to the linear amide. No further research was performed to investigate the proposed

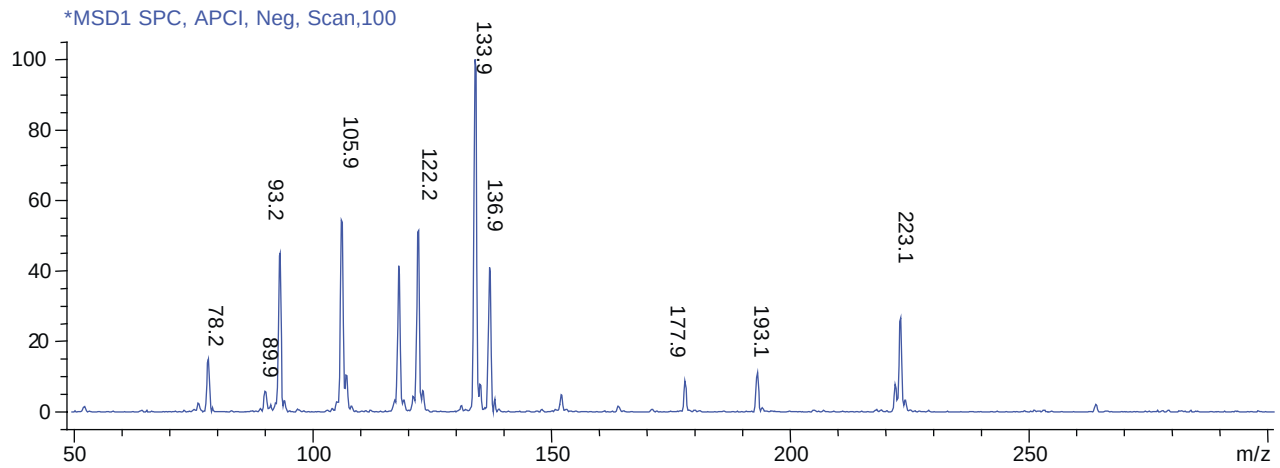

Fig. 16. APCI(−)-MS spectrum of unknown compound at 100 fragmentor voltage.

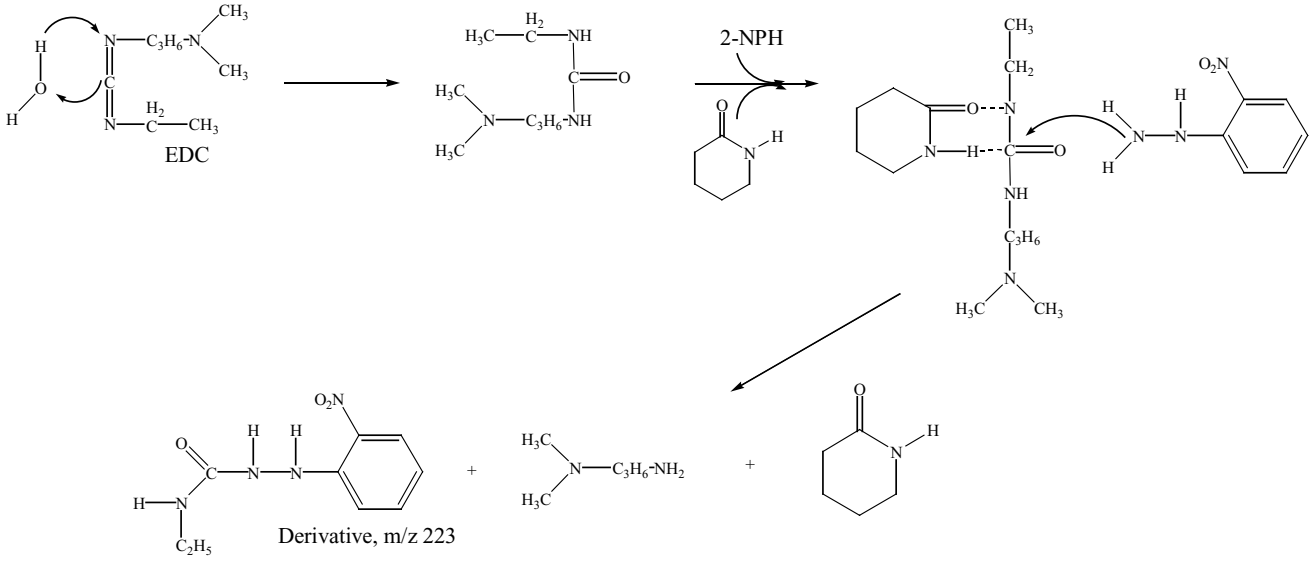

Fig. 18. Supposed reaction mechanism between EDC and 2-NPD, catalysed by cyclic amide.

reaction mechanism, but this specific reaction could be used to determine specifically cyclic amides in certain samples.

4. Conclusion

Carboxylic acids, ketones and aldehydes can selectively and at trace levels be analysed in an aqueous environment by on-line derivatization with 2-NPH with UV detection at $\lambda =$ 400 and 445 nm. The described method shows a linear response in the concentration range between 50 μg/l–10 mg/l and slowly decreasing molar response factors with molecular weight, so a molar response function for the quantification of carboxylic acids, ketones and/or aldehydes can be used for semi quantitative estimation of the concentration. The identification of the derivative can be carried out by their retention times and/or the UV spectrum. The identification of different unknown 2-NPH derivatives of carboxylic acids, ketones and/or aldehydes, in industrial or biological samples, based on retention time and/or UV spectrum is not sufficient. These unknown compounds can be identified with on-line APCI(−)-MS, where identification is based on the molecular mass or/and the fragmentation of the derivative. The investigated fragmentation pattern of different 2-NPH derivatives of carboxylic acids, aldehydes and ketones can be used for structural elucidation. A side product from reaction of EDC and 2-NPH specifically catalysed by cyclic amide could be elucidated by using the described on-line APCI(−)-MS analysis. This specific reaction could be used to determine specifically the presence of cyclic amides in certain samples.

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