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Analysis of keto acids as their methyl esters of 2,4-dinitrophenylhydrazone derivatives by gas chromatography and gas chromatography–mass spectrometry

Rafael Navarro-González*,☆, Alicia Negrón-Mendoza and Guadalupe Albarrán

Instituto de Ciencias Nucleares, UNAM, Circuito Exterior, CU, A. Postal 70-543, México, D.F. 04510 (México)

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ABSTRACT

The analysis of keto acids via reaction with 2,4-dinitrophenylhydrazine (DNP) and esterification with methanol–hydrochloric acid by gas chromatography and gas chromatography–mass spectrometry is described. The derivatives formed (DNPH) are moderately stable and are easy to analyse. The separation of eighteen biologically important keto acids is described. The utility of the method in biochemical studies is demonstrated by the facility of the analysis of keto acids from a plant extract of *Cnidoscolus urens*. A set of electron impact mass spectra of DNPH derivatives is presented.

INTRODUCTION

The analysis of keto acids is an important task in biochemical studies on cellular metabolism. Keto acids are formed as intermediates during metabolic inteconversions of sugars, carboxylic acids and amino acids. Several methods have been developed for the analysis of keto acids using certain types of derivatization [1–9]. Among them, the most widely employed is derivatization via dinitrophenylhydrazine (DNP) [10].

The 2,4-dinytrophenylhydrazone (DNPH) derivatives of keto acids can be used successfully for the isolation of these carboxylic acids from complex mixtures [11]. They have been analysed by spectroscopic techniques [11,12], paper chromatography [13], thin-layer chromatography [14,15] and high-performance liquid chromatography [4,16–18]. Direct separation of DNPHs by gas chromatography (GC) has been accomplished after converting them into methyl esters [19,20]. However, it must be pointed out that in all these chromatographic methods, the separations were complicated as some DNPHs exist as *syn–anti* isomers [13].

This paper describes a method for the analysis of a large number of keto acids as their methyl esters of DNPH derivatives by GC and GC mass spectrometry (MS). The electron impact mass fragmentation pattern of DNPH derivatives is examined, and a set of spectra is provided.

EXPERIMENTAL

Reagents

Keto acids were purchased from Sigma and DNP from Merck. Acetylsuccinic acid was synthesized as its dimethyl ester. Other reagents and solvents were

☆ Present address: Laboratory of Chemical Evolution, Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA.

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of the highest purity commercially available, and were used as received.

Derivatization of standard keto acids

DNPH derivatives were prepared via interaction of the keto acid (0.3 mmol) and DNP (0.5 mmol) in 15% aqueous perchloric acid (30 ml). The mixture was allowed to stand for 30 min at 20°C, then the derivatives were extracted with ethyl acetate (12 ml) by shaking for 3 min three times. The organic phase was evaporated to dryness and the residue was dissolved in 1.6 *M* hydrochloric acid in methanol (10 ml). Esterification was performed by heating the sample in a bath at 70°C for 1 h. After cooling, chloroform–water (4:1) (5 ml) was added and the DNPH derivatives were recovered in the organic phase. The products were recrystallized from benzene or ethyl acetate, and then analysed by GC, GC–MS and infrared spectrometry. This procedure is not affected if esterification is first performed followed by derivatization via DNP.

Derivatization and extraction of keto acids from biological material

Cnidoscolus urens, a typical plant from the Pedregal de San Angel on the south side of Mexico City, was used for this study. Leaves from *Cnidoscolus urens* were blended in doubly distilled water (pH 2–3) with a mixer immediately after harvesting. The homogenate was filtered and allowed to react with DNP as described in the previous section. After reaction, the DNPH derivatives of keto acids and other carbonyl compounds were extracted with ethyl acetate. The organic phase was washed three times with 1 *M* sodium carbonate (in a 3:1, v/v ratio) in order to extract the keto acids selectively. The aqueous phase was then acidified to pH 4–6 with concentrated hydrochloric acid and the keto acids were extracted with ethyl acetate. The organic layer was evaporated to dryness and subsequently esterified in 1.6 *M* hydrochloric acid in methanol (10 ml) as described in the previous section.

GC analysis

A Varian Aerograph Series 2400 gas chromatograph equipped with a flame ionizing detector and a Hewlett-Packard Model 3388A integrator terminal were employed. The chromatographic separation was carried out in a stainless-steel column (1.22 × 32 mm I.D.) packed with 3% OV-17 on Chromosorb W AW DMCS (80–100 mesh). The column temperature was programmed from 200 to 300°C at 6°C/min. The carrier gas was nitrogen at a flow-rate of 33 ml/min. The injector temperature was kept at 250°C. In order to increase the response and reproducibility of DNPH derivatives of keto acids, the detector temperature was maintained at 240°C. This temperature was based on the results of Papa and Turner [21], who found that a lower detector temperature produces an increased response and linearity, improved reproducibility and disappearance of extraneous peaks which are believed to be due to decomposition products of DNPH derivatives of aldehydes and ketones.

GC–MS analysis

GC–MS analysis was carried out on a Hewlett-Packard Model 5890 gas chromatograph coupled to a Hewlett-Packard Series HP-5970 mass detector operated at 70 eV. A PH-1 capillary column from Hewlett-Packard (12 m × 0.2 mm I.D.; film thickness 0.33 μm) was used. The carrier gas was helium at a flow-rate of 2 ml/min. The column temperature was programmed from 150 to 210°C at 6°C/min.

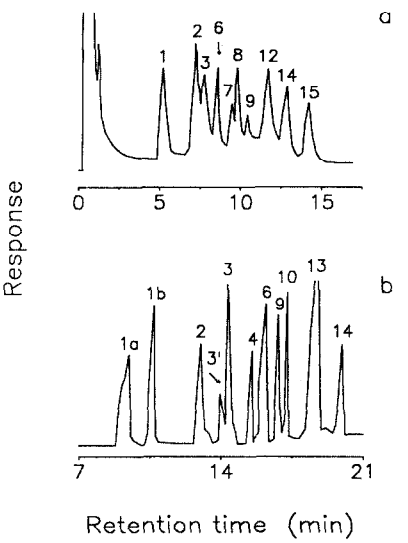


Fig. 1. Gas chromatograms of methyl esters of DNPH derivatives of keto acids: (a) 3% OV-17; (b) HP-1 column. Peak assignments as in Table I.

MS analysis

A VG Analytical Model 7070E mass spectrometer operated at 70 eV was used.

RESULTS AND DISCUSSION

The separation of eighteen biologically important keto acids was investigated. Table I summarizes their retention times for an OV-17 packed column and an HP-1 capillary column. In addition, Table I gives information concerning the molecular weights (MW) of the derivatives and the location of their mass spectra. The chromatographic separations for a variety of keto acids are shown in Fig. 1a and b for an OV-17 and a HP-1 column, respectively.

The method presented facilitates the examination of a wide variety of keto acids in a relatively short chromatographic time of less than 20 min. The detection limit for the determination of keto acids derivatives was established to be in the nanomole range.

Syn–anti isomers and their resolution have been reported in all chromatographic methods where keto acids are converted into DNPH derivatives [4,11–20]. In order to demonstrate whether or not

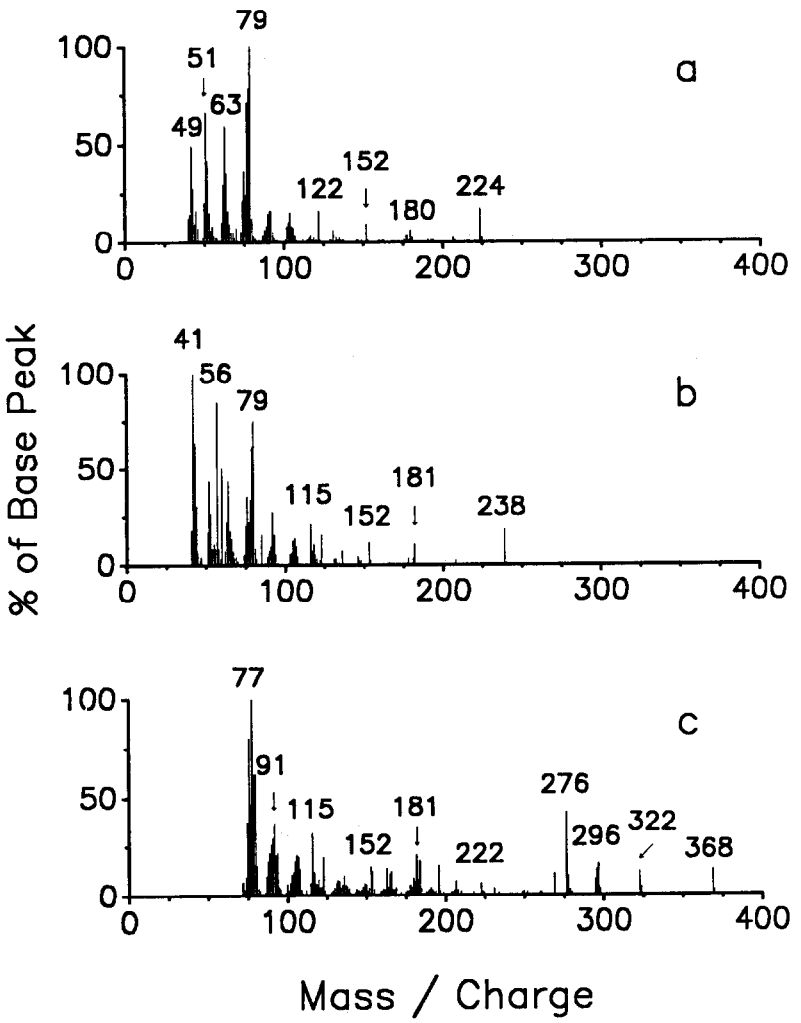
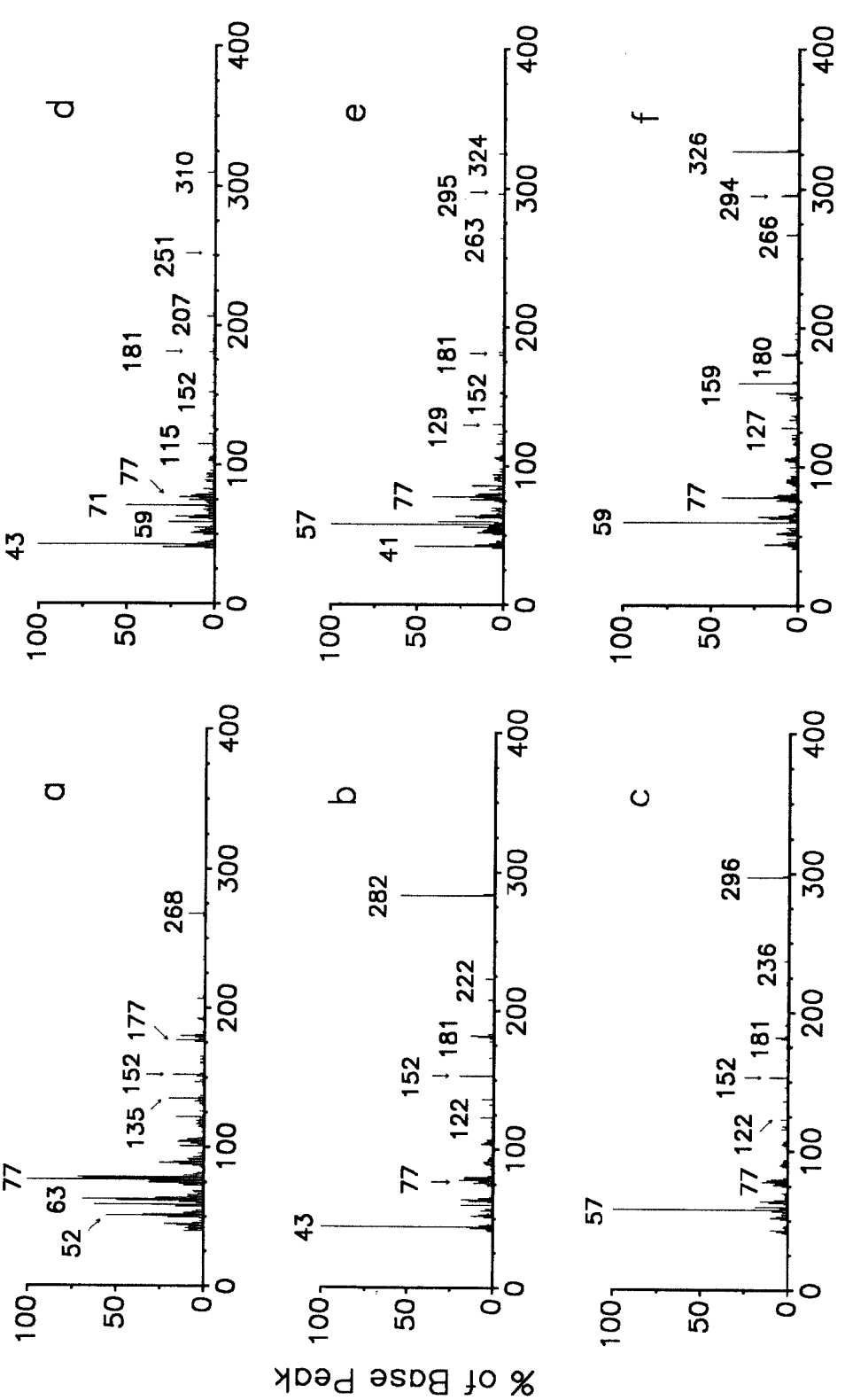


Fig. 2. Mass spectra of methyl esters of DNPH derivatives of β-keto acids: (a) acetoacetic acid, peak 1a; (b) acetoacetic, peak 1b, and β-ketoglutaric acids; (c) oxaloacetic acid (ethyl ester).


a
b
c
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% of Base Peak
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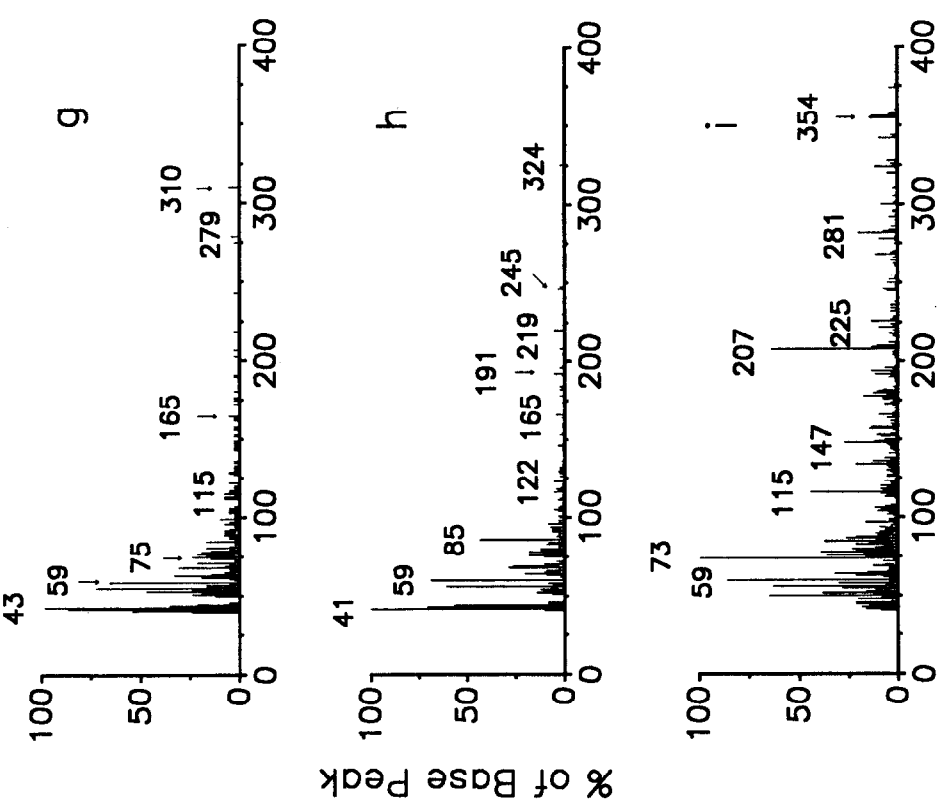

Fig. 3. Mass spectra of methyl esters of DNPH derivatives of keto acids: (a) glyoxylic acid; (b) pyruvic acid; (c) α-ketobutyric acid; (d) α-ketoisovaleric acid; (e) *d,l*-α-keto-β-methyl-*n*-valeric acid; (f) ketomalonic acid; (g) levulinic acid; (h) 4-acetyl-*n*-butyric acid; (i) α-ketoglutaric acid.

TABLE I

RELATIVE RETENTION TIMES OF METHYL ESTERS OF DNPH DERIVATIVES OF KETO ACIDS[a]

Peak No.	Keto acid	MW	Relative retention time		MS (Fig. No.)
			OV-17	HP-1	
1	Acetoacetic	296	0.7	0.7; 0.8	2a, 2b
2	Glyoxylic	268	1.0	1.0	3a
3	Pyruvic	282	1.1	1.1	3b
4	α-Ketobutyric	296	1.1	1.2	3c
5	α-Ketoisovaleric	310	1.2	–	3d
6	*d,l*-α-Keto-β-methyl-*n*-valeric	324	1.9	1.25	3e
7	Acetylsuccinic	368	1.95	–	
8	Acetopyruvic	296	1.4	–	
9	Ketomalonic	326	1.5	1.9	3f
10	Levulinic	310	1.5	1.95	3g
11	β-Ketoadipic	368	1.55	–	
12	Oxaloacetic	340	1.6	1.4	2c
13	4-Acetyl-*n*-butyric	324	1.7	1.45	3h
14	α-Ketoglutaric	354	1.8	1.5	3i
14a	β-Ketoglutaric	354	–	0.8	2b
15	α-Ketoadipic	368	1.9	–	

[a] Normalized to glyoxylic acid.

syn and *anti* isomers are resolved under the chromatographic conditions used here, it was necessary to separate each isomer of glyoxylic and pyruvic acid DNPHs according to the method of Katsuki *et al.* [11]. Each isomer was checked by spectroscopic measurements (300–500 nm) and the results obtained were similar to those of Katsuki and coworkers [11,12]. After esterification, the isomers were analysed by GC. The results showed that both isomers have equal retention times, and when *syn* and *anti* isomers were co-injected, they appeared as a single peak on an OV-17 column. Resolution of *syn–anti* isomers was only observed for pyruvic acid on an HP-1 column. This finding was further confirmed by GC–MS, where the spectra of both isomers (peaks 3′ and 3 in Fig. 1b) were identical.

The DNPH derivatives of hydroxypyruvic and oxalomalic acid could not be analysed by this technique as their melting points were higher than 300°C. The high melting point for hydroxypurivic acid DNPH is in agreement with the expected structure derived from the reaction of the acid with DNP, as an α-ketol structure undergoes double addition of DNP, leading to the formation of an osazone [22].

The analysis of β-keto acids was complicated owing to their instability in an acid medium leading to their decarboxylation. Table II summarizes the decarboxylation products for some unstable methyl ester DNPH derivatives of keto acids. For instance, decarboxylation of acetoacetic acid resulted in the formation of 2,4-dinitrophenylhydrazone derivatives of acetaldehyde and acetone, peaks 1a and 1b in Fig. 1, respectively. The identification of these compounds was confirmed by mass spectrometry (Fig. 2a and b); these spectra are identical with pub-

TABLE II

DECARBOXYLATION PRODUCTS OF SOME UNSTABLE METHYL ESTERS OF DNPH DERIVATIVES OF KETO ACIDS

Keto acid	Product[a]	Extent of decomposition
Acetoacetic	Acetaldehyde, acetone	Complete
Ketomalonic	Glyoxylic acid	Partial
Oxaloacetic	Pyruvic acid	Complete
β-Ketoglutaric	Acetone	Complete

[a] Detected as DNPHs.

lished mass spectra [23]. In a similar fashion, oxaloacetic decomposed completely into pyruvic acid. However, if the reaction of DNP is done with oxaloacetic acid diethyl ester, partial decarboxylation takes place. Fig. 2c shows the mass spectrum of the ethyl ester of the DNPH of oxaloacetic obtained directly from the crystallized product. The spectrum shows the presence of the molecular ions for oxaloacetic acid (MW 368) and pyruvic acid (MW 296).

The reaction of DNP with β-keto acids may lead to the formation of a cyclic structure (pyrazolones) [22]. Under our experimental conditions, the formation of such cylic compounds did not occur. However, some reported mass spectral data [20] indicate the existence of both linear and cyclic structures for oxalocetic acid.

Mass spectra for a variety of keto acids are given in Fig. 3. The masses of major fragmentation peaks characterizing the compounds are indicated. Common fragments for keto acids are at masses 63, 75, 77, 79, 91, 105, 127, 152 and 180. Most of the peaks

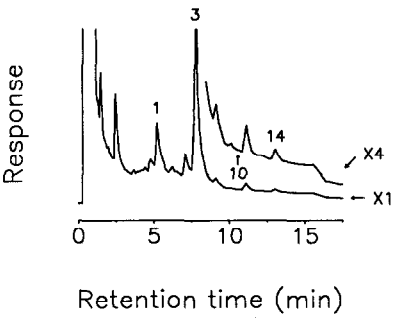

Fig. 4. Relationship between intensity of molecular ion peak and total number of carbon atoms present in the derivative. ○ = α-keto acid; △ = α-keto dicarboxylic acid; ▽ = γ-keto acid; □ = δ-keto acid.

in the lower part of the spectra are due to the DNP moiety, and are also characteristic of DNPHs of aldehydes and ketones [23].

The molecular ion (MI) peak was present in all instances. Its intensity was found to depend on the total number of carbon atoms present in the derivative (Fig. 4). The maximum intensity was pro-

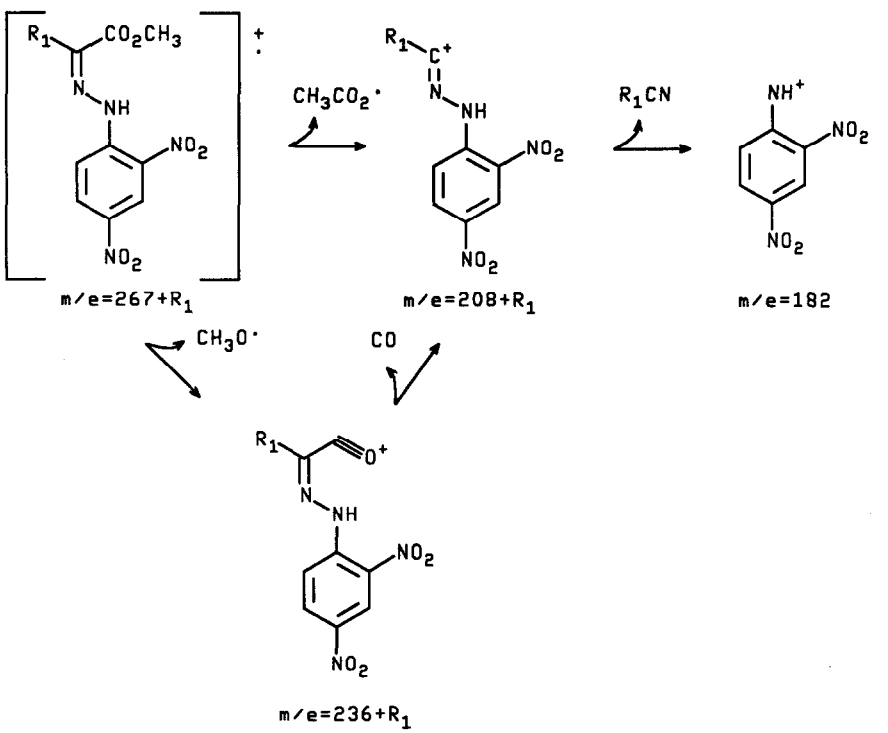

Fig. 5. Fragmentation pattern of methyl esters of DNPH derivatives of α-keto acids.

duced with pyruvic and ketomalonic acid (55% and 39%, respectively).

Characteristic peaks in the mass spectra of keto acids are at masses 55, 57 and 73 and at MI − 31, MI − 59, MI − 60 and MI − 73. These peaks are usually smaller than the peak at mass 59, and can be used as specific signs for identification of these compounds. Based on the MS data, a fragmentation pattern for methyl esters of the DNPHs of α-keto acids is given in Fig. 5.

The method presented for the detection, separation and identification of keto acids has the following advantages: (1) low GC and GC–MS running time; (2) good separation for a wide variety of keto acids; (3) no resolution of *syn–anti* isomers; (4) high detection sensitivity; and (5) ease of identification by MS. Therefore, this method can be adopted in studies of amino acid catabolism, maple syrup urine disease, diabetes and other metabolic or chemical studies. The adaptability of the method to biological material is illustrated by the analysis of keto acids present in leaves of *Cnidoscolus urens* (Fig. 6).

One disadvantage of the technique, which applies to all chromatographic separations of keto acids utilizing derivatization via DNP, is the decarboxylation of β-keto acids.

CONCLUSIONS

Derivatization of keto acids via DNP has been used successfully for their isolation from complex mixtures; however, it has been frequently criticized for imperfect qualitative and quantitative chromatographic analyses owing to the resolution of *syn* and *anti* isomers. This resolution leads to a greater number of peaks in the chromatogram and complicates the identification of keto acids [10,12]. The present method represents a definite improvement for the analysis of keto acids as there is no longer the problem of *syn* and *anti* isomer resolution. In addition, it offers the advantage of analysing a wide variety of keto acids in a short chromatographic time with a detection limit in the nanomole range.

The electron impact mass spectra for ten keto acids are provided. In addition, a mechanism for the fragmentation of methyl esters of the DNPHs of α-keto acids is presented.

This technique is now used routinely in our laboratory for the search for keto acids formed during the γ-radiolysis of aqueous solutions of organic compounds.

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