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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SOME CARBOXYLIC ACIDS IN FOOD AND BEVERAGES AS THEIR *p*-NITROBENZYL ESTERS

R. BADOUD* and G. PRATZ
Nestec Ltd., Nestlé Research Centre, CH-1800 Vevey (Switzerland)
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SUMMARY

An improved, straightforward and accurate method for the derivatization and quantitative high-performance liquid chromatographic analysis of carboxylic acids in food and beverages is presented. Thirty-two *p*-nitrobenzyl esters were prepared in high yields by direct reaction of the free acid with O-(4-nitrobenzyl)-N,N′-diisopropylisourea in dioxane–water (9:1). Benzylmalonic acid was used as a new internal standard. Excess of reagent was removed on a strong cation-exchange resin. The advantage of this original and very convenient procedure over the usual clean-up step involving filtration through a disposable silica cartridge is discussed. The *p*-nitrobenzyl ester derivatives were readily separated using a single linear gradient of solvent (acetonitrile in water). With a high-performance liquid chromatography column of conventional size (20–25 cm × 4.6 mm I.D.), the analysis lasted for less than 20 min. This analysis time was even shorter (< 12 min) when using modern small-bore (100 × 2.1 mm I.D.) columns. Applications of the method to the analysis of the main carboxylic acids in coffee, wine and fruit juices are presented.

INTRODUCTION

The determination of carboxylic acids in food and beverages by high-performance liquid chromatography (HPLC) is already well documented[1–11]. According to some authors[10], four main methods are commonly used, namely ion-exchange and ion-exclusion chromatography, separation based on solvophobic interactions, ion-pair chromatography and reversed-phase separation of derivatized products.

The choice of the method depends on many factors. These include the nature of the acids to be analysed (volatile, aromatic, polyfunctional) and of the matrix in which they are present (fruit juices, fermentation products), as well as their relative concentration in the sample and the complexity of the latter. In most cases, the carboxylic acids need to be converted to the corresponding ester derivatives to enhance their detectability by light absorbtion. Thus, the selection of a suitable labelling group is also decisive.

Many chromogenic and fluorogenic groups have been developed to tag car-

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boxylic acids for HPLC analyses in the reversed-phase mode. These are principally substituted phenacyl[10,12–17] and naphthacyl[18] groups, the *p*-nitrobenzyl (PNB)[19–24] group, 4-methyl-7-methoxy-[25,26] or 4-methyl-6,7-dimethoxycoumarin derivatives[27], as well as the 9-methyl-anthracene[28,29] group.

Up to now, the carboxylic acids present in green or roasted coffee have been chiefly determined either by gas chromatography (GC) or isotachophoresis[30,31]. Both methods are very useful but have their limitations. For instance, isotachophoresis is not commonly found in every laboratory, and GC needs volatile compounds and extensive clean-up of the sample. Hence, there is still a need for a straightforward method using conventional HPLC equipment that could be proposed as a true alternative.

The aim and purpose of the development of the reported procedure was thus to find a suitable HPLC analytical method to quantitate with accuracy the major carboxylic acids present in coffee and in other beverages. The main requirement was to keep the sample treatment as simple as possible both to avoid the potential formation of artefacts and to save time and cost per analysis. Coffee already contains many UV absorbing components, such as the well-known chlorogenic acids (caffeoylquinic esters and parent compounds), so a suitable chromophoric labelling that could specifically reveal aliphatic carboxylic acids in the presence of other UV absorbing species was needed.

The analytical method reported here uses the HPLC separation of PNB ester derivatives. The improved preparation and separation of these compounds, the definition of a suitable internal standard, and problems related to the clean-up operation prior to HPLC analysis will be discussed.

EXPERIMENTAL

Chemicals and supplies

Aliphatic carboxylic acids (or their sodium salts) were purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, F.R.G.). These were of the highest purity available and were not further purified. The strong cation-exchange resin Dowex 50W-X8 (100–200 mesh, *p.a.*) was obtained from Fluka. Acetonitrile (Romil Chemicals, by Amman-Technik AG, Kölliken, Switzerland) was redistilled before use and 1,4-dioxane (Merck, *p.a.*) was redistilled over sodium. HPLC ultrapure water was generated by a Milli-RO4 coupled to a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). All solvents were filtered through 0.45 μm membranes (Millipore). O-(4-Nitrobenzyl)-N,N′-diisopropylisourea (PNBDI) was synthesised according to Schmidt *et al.*[32] and purified by repeated recrystallization from pentane. The reagent is also commercially available (Fluka; Pierce, Rockford, IL, U.S.A. or Regis, Morton Grove, IL, U.S.A. through Chemie Brunschwig, Basel Switzerland), but it is fairly expensive (Fluka: US $ 12.00/100 mg) and generally needs to be purified further. All samples were filtered through 0.2-μm disposable filters (Acro LC13, Gelman Sciences, Ann Arbor, MI, U.S.A.) before injection.

Standard solutions

Stock solutions of the standards were prepared from the free acids (except lactic and phosphoric acids which were a sodium lactate and a sodium dihydrogen-

phosphate solution) at a concentration of 100 ± 1 μmol/ml in water (aliphatic acids) or in water–methanol mixtures (aromatic acids). Benzylmalonic acid in water (100 μmol/ml) was used as the stock internal standard (I.S.) solution. Five working standard mixtures were prepared by mixing equimolar quantities of the stock solutions and of the internal standard to make 10 μmol/ml (except mixture I, 9.1 μmol/ml) of each compound after dilution. Mixture I, quinic, glycolic, pyroglutamic, lactic, formic, acetic, tartaric, malic, phosphoric, citric, I.S.; mixture II, malonic, maleic, itaconic, glutaric, fumaric, I.S.; mixture III, furan-2-carboxylic, succinic, citraconic, mesaconic, (nicotinic), I.S.; mixture IV, tartronic, propionic, butyric, sorbic, I.S.; mixture V, gentisic, mandelic, benzoic, phenylacetic, I.S.

Sample preparation

Coffee samples. To 0.5 g of instant coffee powder in a 25-ml volumetric flask approximately 15 ml of bidistilled water and 750 μl of a 0.1 *M* aqueous solution of benzylmalonic acid (I.S., final concentration = 3 μmol/ml) were added. Alternatively, extracts were prepared using aqueous 5 m*M* benzylmalonic acid instead of water. After shaking the mixture in an ultrasonic bath for 10 min at room temperature, and making up the flask to volume, the resulting suspension was filtered (filter paper) or centrifuged. A 4–5-ml aliquot was treated (gentle shaking) for a few minutes with 0.5 g of Dowex 50W-X8. A portion of the clear supernatant (50 μl) was used for the subsequent derivatization. Samples of ground coffee were similarly prepared.

Samples of wine or fruit juice. These were filtered and diluted with water if required. Typically, the sample was then mixed with 0.1 *M* aqueous benzylmalonic acid (I.S.) in the ratio of 9:1 and a portion of the resulting mixture treated with the cation-exchange resin (0.1 g/ml) as above. All the standard mixtures underwent the same type of treatment.

Derivatization

The sample (or standard) solutions (50 μl) were placed in 3.5-ml PTFE-lined screw-capped amber vials. A freshly prepared solution of 10 mg of PNBDI in 500 μl of dioxane (redistilled over sodium) was added. The vials were immediately well-stoppered and placed in a heating dry-block for 60 min at 80°C. The reaction mixtures were then cooled down and cleaned-up according to either procedure A or B.

In a separate series of experiments, 50 μl of standard mixture I were derivatized as above except that the solvent was either dichloromethane, acetonitrile or THF (redistilled over sodium).

Clean-up procedure A[23]. The crude reaction mixture was diluted with 8 ml of dichloromethane and passed through a disposable pre-conditioned silica clean-up cartridge (Waters Sep-Pak). An additional 8 ml of dichloromethane was used to rinse the support. The combined eluates were carefully evaporated to dryness, the residue redissolved in acetonitrile (2.5 ml) and the resulting solution filtered through a 0.2-μm liquid chromatographic (LC) filter. Bond-Elut silica cartridges (100 mg adsorbent; Analytichem) were also used: the reaction mixture was diluted in 4 ml of dichloromethane then placed on the top of the cartridge and eluted with an additional 10 ml of solvent.

Clean-up procedure B. After cooling, the reaction mixture was diluted by the addition of 2 ml of acetonitrile and 0.5 g (50 mg/mg PNBDI) of Dowex 50W-X8 was

added. The mixture was briefly shaken and left in contact with the resin for at least 15 min before decanting and filtering the supernatant through a 0.2-μm disposable LC filter disk.

Chromatographic equipment

Two liquid chromatographic instruments were used. The first was a HP 1090A liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.) equipped with two delivery pumps, a high-speed 1040A diode-array detector, a column oven and a manually controlled Model 7010 injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 5-μl loop. The instrument was controlled by a HP-85B personal computer linked to a 9121D mass storage unit and a 7470A plotter (Hewlett-Packard). The second chromatograph consisted of two Model M6000A pumps and a Model M45 pump, a Model M720 gradient controller, a Model M730 data module and a Model WISP 710B automated sample injector (Waters Assoc., Milford, MA, U.S.A.). The UV detector was a HP 1040A diode-array detector (Hewlett-Packard) controlled by a HP-85A personal computer linked to a Model 82901M flexible disk drive and a 7470A plotter (Hewlett-Packard). The column was thermostated in a Waters column temperature control module (Cat. No. 07011) and the solvents deaerated with helium using a Spectra-Physics (San José, CA, U.S.A.) manifold accessory. This equipment was completed with a solvent select accessory (Waters, Cat. No. 60021) and an automated switching valve (Waters, Cat. No. 60057).

Integrations, recalculations and data storage were performed with a Model 3357 laboratory automation system (Hewlett-Packard). Derivatizations of the samples were performed in a Pierce (Kontron, Zürich, Switzerland) Reacti-Therm heating dry-block.

Analytical conditions

Two types of RP-18 (5 μm) column (according to their internal diameter) were used. Column A was either a Spheri-5, 220 × 4.6 mm I.D. MPLC cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) or a Nucleosil-5, 250 × 4 mm I.D. column (Macherey-Nagel, Düren, F.R.G.), and column B was a Hypersil ODS, 100 × 2.1 mm I.D. column (Hewlett-Packard). The type A columns were connected to an automated rotary switching valve in such a way that the flow passed through a pre-column (Aquapore RP-300, 30 × 2.1 mm I.D., Brownlee Labs.) which could be back-flushed if needed at the end of the run using an additional pumping system. With the type B column, either the above-mentioned guard-column or a 20 × 2.1 mm I.D. pre-column (Hewlett-Packard) filled with the same material as the analytical column was used in the normal way. In both cases, a high-pressure in-line filter (Hewlett-Packard) containing a frit of 2 μm pore size (1.6 mm diameter) was inserted between the injector and the pre-column. The mobile phase was composed of water (A) and acetonitrile (B). Compounds were eluted using a linear gradient of solvent. Flow-rates were 1 ml/min for type A columns and 0.4 ml/min for type B. Both columns were thermostated at 25°C.

Eluents were detected at 265 nm using a 4-nm bandwidth and a time-base set at 320 ms.

RESULTS AND DISCUSSION

Although O-(4-nitrobenzyl)-N,N′-diisopropylisourea (PNBDI) is a well-known derivatizing agent supplied by several major chemical companies (Fluka, Pierce or Regis), it is probably not as widely used as it might be. In recent years, indeed, very few publications on the use of this reagent have appeared in the literature[19,21–24].

Yet, PNBDI is a powerful reagent that readily reacts with free carboxylic acids to form the corresponding PNB esters in high yields without catalyst in a pure organic or water-containing solvent. It can be conveniently synthesized according to the literature procedure by the addition of PNB alcohol to N,N′-diisopropylcarbodiimide[32] followed by a careful purification. However, it has one major drawback; it is not compatible with the stationary phase of the HPLC column because of reaction with free silanol groups and/or displacement of the grafted alkyl-silane chain which can be washed out, and must be eliminated before injection of the sample. Therefore, when the derivatization reaction is completed, an efficient clean-up step to decompose or remove excess reagent (*vide infra*) should be effected.

Derivatization and clean-up

Samples were derivatized by heating (80°C for 1 h) a mixture of the aqueous sample and a solution of PNBDI in dioxane (1:9). The proportion of reagent to the number of carboxylate groups present in the sample was in the range 2:1 to 30:1. At the end of the reaction time, the mixture was cleaned up to remove any unreacted reagent.

Fig. 1 shows the separation of the PNB esters obtained after the derivatization of an equimolar standard mixture of ten carboxylic acids (numbers refer to compounds listed in Table I). Both chromatograms are recorded at the same detector

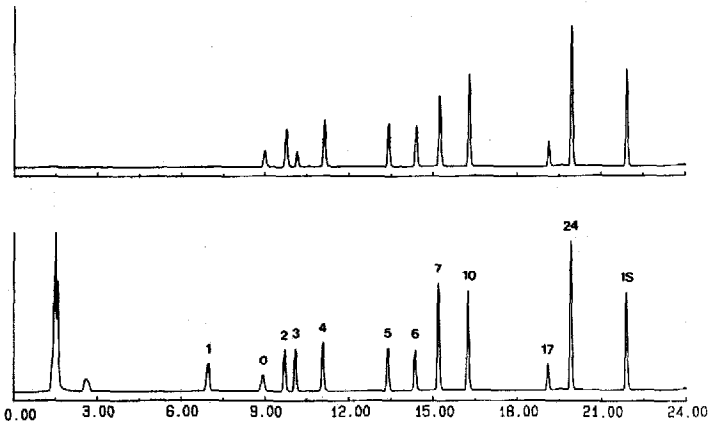

Fig. 1. Separation of the PNB esters obtained after the derivatization of the standard carboxylic acid mixture I. Fifteen microlitres of approximately 0.2 μmol/ml of each acid were injected through a Nucleosil-5 RP-18 (250 × 4 mm I.D.) column. Solvent A was water and solvent B was acetonitrile. The gradient was 20–80% B in 20 min at 1 ml/min. The eluent was detected at 265 nm. Upper trace, after clean-up through Sep-Pak Si cartridge; lower trace, after exposure to Dowex 50W-X8. Numbers correspond to the compounds listed in Table I.

TABLE I

RETENTION TIMES OF THE DERIVATIZED CARBOXYLIC ACIDS

Type A (4.6 mm I.D.) column using linear gradients of solvent: (A) 20–80% acetonitrile in water in 20 min; (B) 10–100% acetonitrile in water in 20 min. ND = Not determined.

No.	*Acid*	t_R *(min)*	
		A	*B*
1	Quinic	7.10	8.60
0	*p*-Nitrobenzoic	9.00	10.15
2	Glycolic	9.75	10.70
3	Pyroglutamic	10.15	10.90
4	Lactic	11.10	11.65
5	Formic	13.45	13.30
6	Acetic	14.40	13.95
7	Tartaric	15.25	14.30
27	Gentisic	15.25	14.50
	Nicotinic	15.50	ND
8	Tartronic	15.75	14.65
9	Mandelic	16.15	15.00
10	Malic	16.30	15.00
11	Propionic	16.50	15.35
	p-Nitrobenzoyl chloride	16.50	15.35
12	Furan-2-carboxylic	16.95	15.55
13	Butyric	18.25	16.50
14	Malonic	18.40	16.40
15	Maleic	18.80	16.70
16	Succinic	18.95	16.80
17	Phosphoric	19.15	16.85
18	Phenylacetic	19.18	17.50
19	Itaconic	19.35	17.10
20	Benzoic	19.75	17.05
21	Glutaric	19.80	17.40
22	Citraconic	19.85	17.40
23	Sorbic	19.85	17.60
24	Citric	19.95	17.40
25	Fumaric	20.25	17.65
26	Mesaconic	21.05	18.40
	2-Naphthylacetic	21.15	ND
	Diphenylacetic	21.40	ND
	Cyclohexanecarboxylic	21.75	ND
I.S.	Benzylmalonic	21.90	18.75

amplitude (265 nm). The upper chromatogram shows the separation of the derivatization mixture that was cleaned up following an already-known procedure[2,3]. This was accomplished by passing the crude reaction mixture (previously diluted in dichloromethane) through a disposable silica cartridge (Waters Sep-Pak) that retained PNBDI and the by-product N,N′-diisopropylurea. The lower chromatogram shows the separation of the same reaction mixture, which after dilution with acetonitrile was simply exposed to the strong cation-exchange resin (clean-up procedure B). The former operation was not only costly and time-consuming, but more importantly, it totally removed quinic acid PNB ester (peak no. 1) and partially eliminated the de-

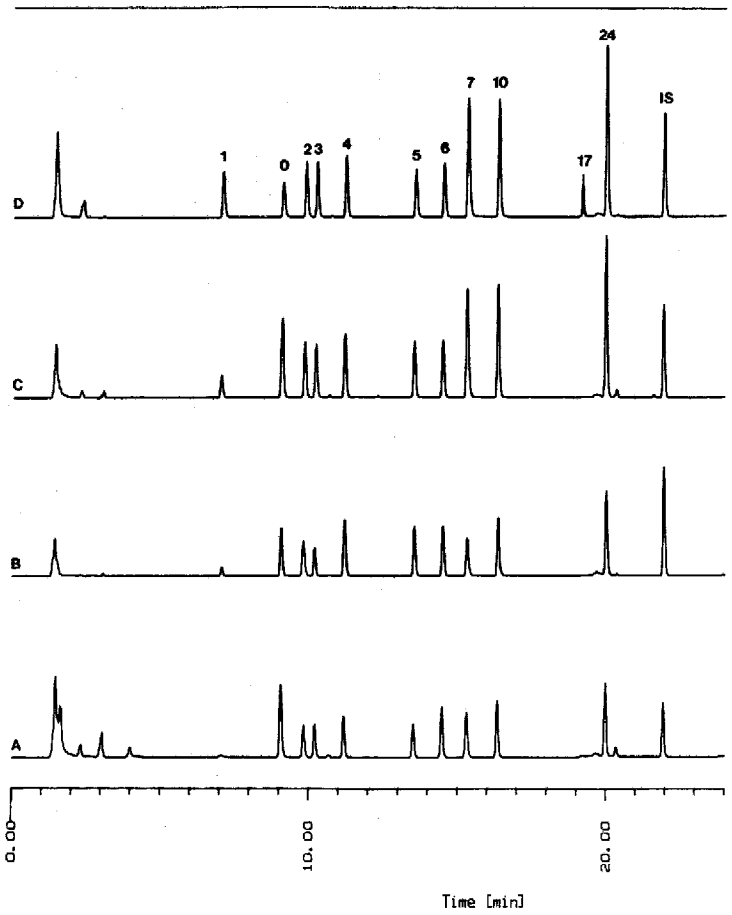

Fig. 2. Influence of the reaction solvent on the relative derivatization yields of the standard acids in mixture I. Trace A, acetonitrile; trace B, dichloromethane; trace C, tetrahydrofuran; trace D, 1,4-dioxane. The analytical conditions were the same as for Fig. 1.

rivatives of pyroglutamic (2-pyrrolidone-5-carboxylic, 3) and tartaric (7)* acids.

Unlike silica, the ion-exchange resin apparently did not decompose or retain any PNB-esters. The peak heights in the lower chromatogram were always larger or at least of the same size as compared with the peak heights in the upper plot. It is likely that the sulphonic acid groups of the polymer reacted with the excess of reagent to form the corresponding immobilized PNB ester. This hypothesis was supported by the fact that PNB alcohol was released on heating the resin used to trap the reagent (in acetonitrile or dioxane). This same experiment performed in the presence of free carboxylic acids did not produce any PNB esters. However, the ability of a

* The use of a Bond-Elut silica cartridge gave somewhat better results; see Experimental.

PNB-sulphonic ester resin to react with free carboxylic acids to provide the corresponding PNB esters is worth investigating.

Very recently, after this clean-up procedure was developed, Bandi and Reynolds[24] reported the use of sulphuric acid to destroy the excess reagent.

Furthermore, it was found that not only carboxylic and sulphonic but also phosphoric and nitric groups or halides readily reacted with PNBDI under certain conditions (*vide infra*). Thus, it was possible to simultaneously analyse phosphoric acid in the form of its tris-(*p*-nitrobenzyl)-phosphate (peak no. 17). Later, the identity of this product was confirmed by comparison of its retention time (t_R) with that of an authentic compound independently synthesized[33]. Although the formation of this derivative was far from being quantitative, it was sufficiently reproducible to allow its determination in coffee.

Choice of the solvent for the derivatization reaction

The formation of the phosphoric acid PNB ester and also of the carboxylic PNB esters was strongly solvent dependent. Chromatograms A–D of Fig. 2 (relative plots) show the separation of the PNB esters obtained after the derivatization (1 h at 80°C) of a standard mixture of carboxylic acids (mixture no. I; containing phosphate ions) in acetonitrile (A), dichloromethane (B), tetrahydrofuran (C) and dioxane (D). The latter gave the best results. The relative yield of quinic acid PNB ester was strongly enhanced (peak no. 1 was bigger in D than in C) as compared with the results obtained in THF, the solvent used by Steiner *et al.*[23]. Furthermore, the formation of the PNB ester of phosphoric acid (peak no. 17) was only observed when dioxane was used.

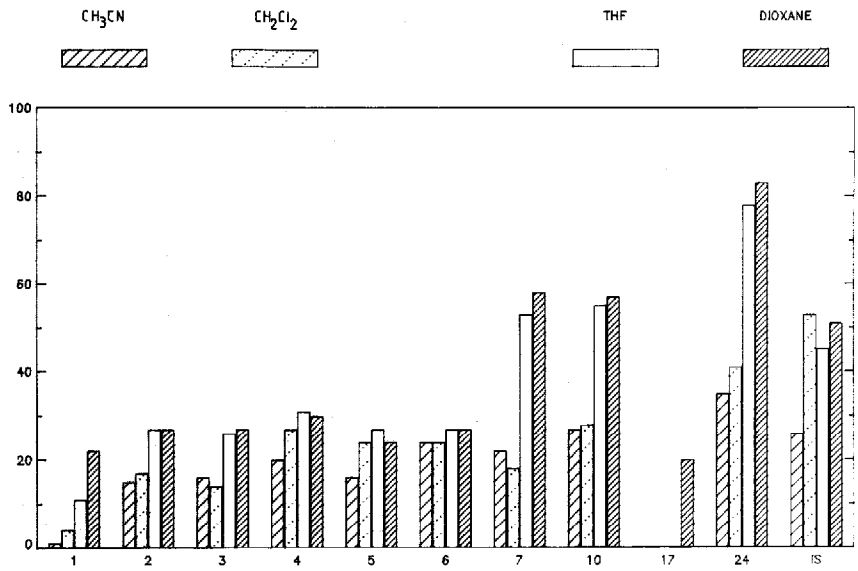

Fig. 3. Relative peak heights (arbitrary units) of the PNB esters as measured after the injection of equal volumes of derivatization mixtures prepared in four different solvents (acetonitrile, dichloromethane, THF and dioxane). Numbers on the abscissa refer to the carboxylic acids of Fig. 2.

Actual yields for the derivatization of each acid were not determined. However, knowing that the standard acids were prepared as an equimolar mixture and assuming that the extinction coefficients were more or less equal in magnitude and additive, the heights of the peaks should correspond (phosphoric acid excepted) to the number of carboxylate groups present in each molecule. Indeed, peak-height ratios corresponding to the PNB esters in chromatogram D (Fig. 2) are remarkably close to those expected. This suggests that yields are equally high for all the carboxylic acids tested. The relative peak heights are reported in Fig. 3, which clearly demonstrates the superiority of dioxane as the derivatization solvent. The same observations also apply for the other standard carboxylic acid mixtures, the separations for which are shown in Figs. 4–8.

Unfortunately, two important compounds, oxalic and pyruvic acids, did not give characterizable derivatives and could not be quantified by the present procedure. However, good and selective derivatization methods (formation of fluorescent quinoxaline derivatives) already exist for them and for other α-oxo-carboxylic acids. Caffeic acid and related compounds or chlorogenic acid isomers (of particular concern for the coffee samples) did not provide a single derivatization product either. However, in this case, the different behaviour of such compounds enhanced the selectivity of the method.

Chromatographic separation

The PNB ester derivatives were separated in reversed-phase mode through a C_{18} column using a linear gradient of solvents composed of water (A) and acetonitrile (B). For the sake of clarity, and because not all the compounds could be simultaneously separated with the same elution programme, five different mixtures of standard acids (mixtures I–V) were derivatized and analysed. The separations of these are shown in Figs. 4–8. In these cases, the HPLC conditions were not the same as in

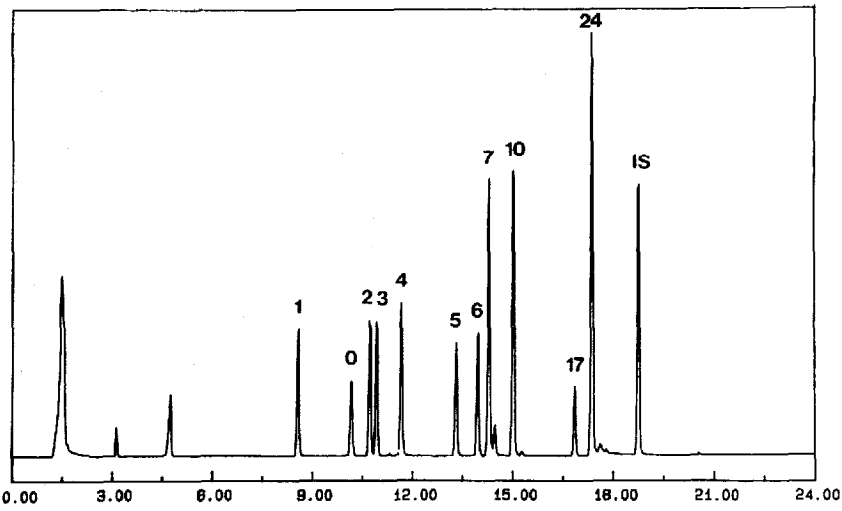

Fig. 4. Separation of the PNB esters of mixture I using the same analytical conditions as previously, except that the gradient was 10–100% B in 20 min.

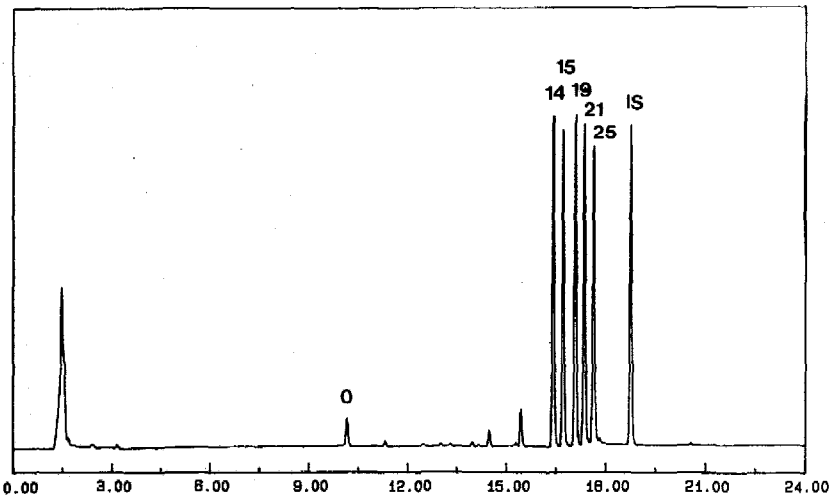

Fig. 5. Separation of the PNB esters of mixture II. The analytical conditions were the same as in Fig. 4.

Figs. 1 and 2. The compounds were now separated in less than 20 min with a linear gradient of 10–100% B in 20 min (flow-rate 1 ml/min). Their corresponding retention times (t_R), as determined under two different analytical conditions, are reported in Table I. They proved to be very reproducible (± 0.05 min) over long periods of time (several weeks) provided that the solvent delivery system was well tuned and the temperature carefully controlled (see *Equipment*). A few interferences could not be eliminated by a simple adjustment of the gradient slope. However, no real sample

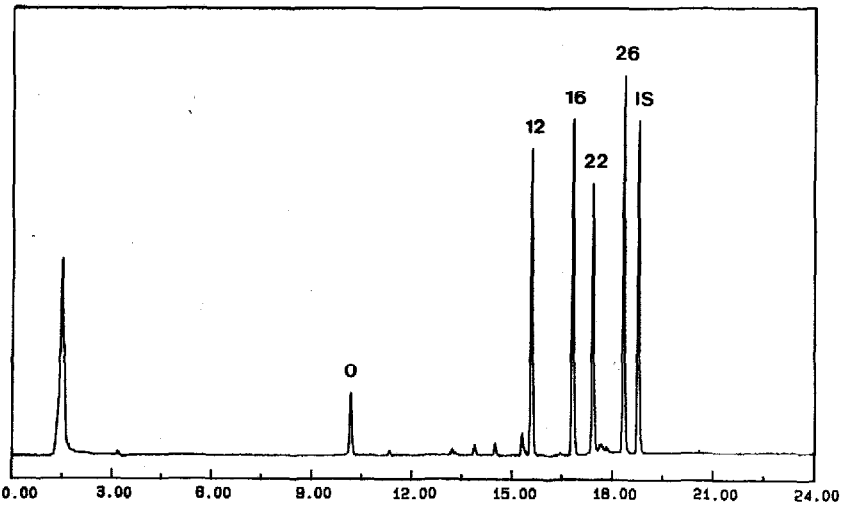

Fig. 6. Separation of the PNB esters of mixture III. Nicotinic acid present in the original mixture was retained by the cation-exchange resin during the pre-treatment step of the sample and is not seen (*cf.* with Fig. 11).

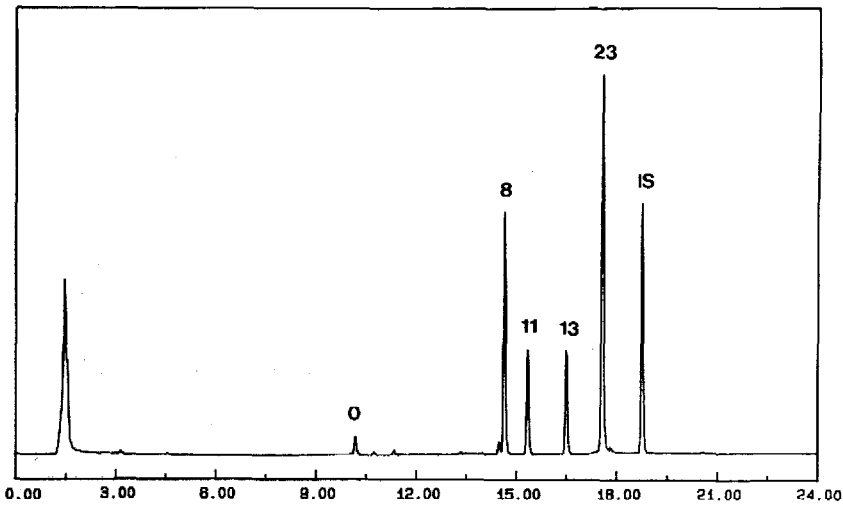

Fig. 7. Separation of the PNB esters of mixture IV. The analytical conditions were the same as in Fig. 4.

contains all of these carboxylic acids at the same time. Therefore, the resolution was good enough in most cases.

By using short small-bore columns (100 × 2.1 mm I.D.) the analysis time could be reduced further. As an illustration, the separation of standard mixture I is shown in Fig. 9. A complete separation was achieved in less than 12 min using a steep gradient (0–70% B in 10 min at 0.4 ml/min), with the additional advantage of reducing the solvent consumption. On a general basis, the dimension of the column and hence the gradient, were chosen as a function of the composition (degree of complexity) of the sample to be analysed.

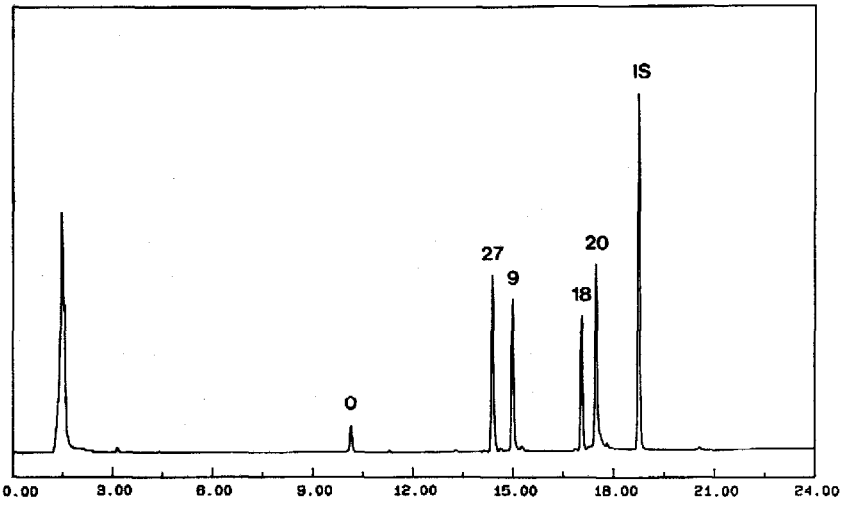

Fig. 8. Separation of the PNB esters of mixture V. The analytical conditions were the same as in Fig. 4.

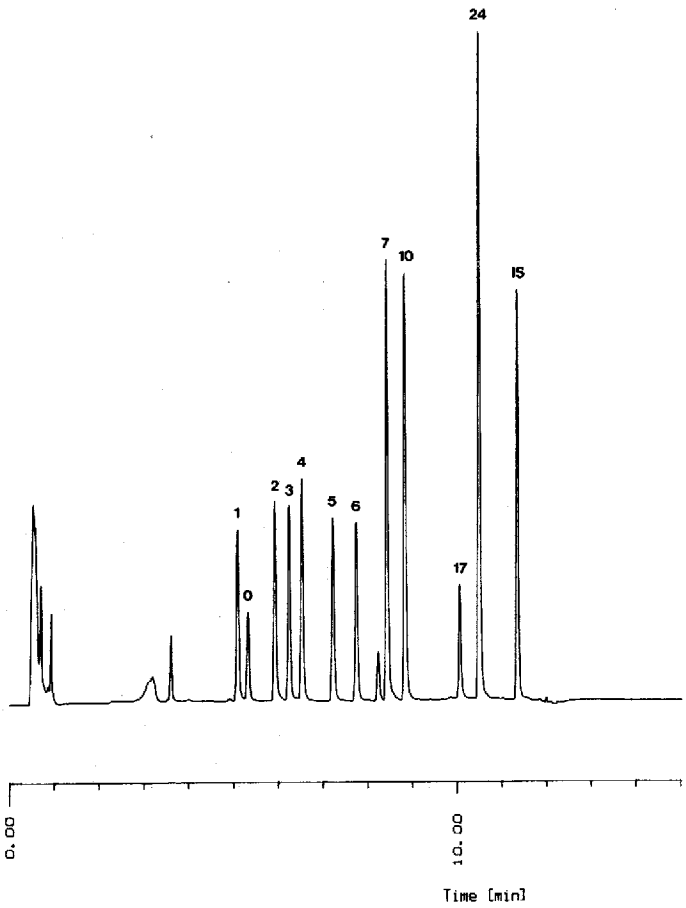

Fig. 9. Chromatographic separation of the PNB esters of mixture I on a 100 × 2.1 mm I.D., 5 μm, RP-18 small-bore column using a solvent gradient of 0–70% B in 10 min. Five microlitres of the derivatization mixture containing 0.2 μmol/ml each acid were injected.

Choice of internal standard

Several carboxylic acids for which PNB esters could act as an internal standard were investigated. They were chosen according to the usual criteria of purity, availability and non-interference with other components of the sample. The following compounds were tested: 2-naphthylacetic, diphenylacetic, 2-naphthoic, cyclohexanecarboxylic and benzylmalonic acids. The last two were retained for two main reasons. First, their PNB ester eluted in a region of the chromatogram free of peaks (see figures and Table I). Secondly, both compounds showed different solubility properties: cyclohexanecarboxylic acid was soluble in organic solvents and could be used for liposoluble materials (long-chain fatty acids for instance), while benzylmalonic acid was water-soluble and could be employed in the analysis of aqueous samples (beverages, coffee extracts).

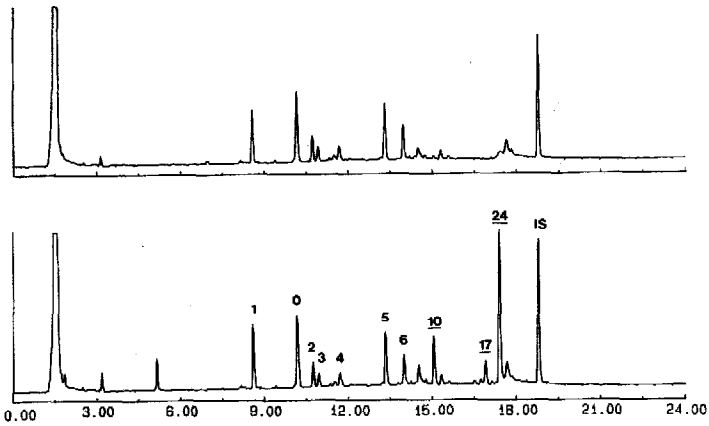

Fig. 10. Separation of the PNB esters obtained after the derivatization of a coffee sample using the same analytical conditions as in Fig. 4. Upper trace, without pre-treatment of the aqueous extract with the cation-exchange resin; lower trace, with the usual treatment.

Role of cation-exchange pre-treatment

Samples of instant or roasted ground coffees were prepared as an aqueous extract (2–5%) to which was added a known amount of internal standard (benzylmalonic acid). The solution was then treated with a strong cation-exchange resin (Dowex 50W-X8) to free the carboxylic acids before derivatization. Omitting this treatment produced an incomplete profile, as seen in Fig. 10. The upper trace of the figure shows the separation of the PNB esters of a coffee sample not treated with the ion-exchange resin. As compared with the lower trace, three PNB esters (underlined

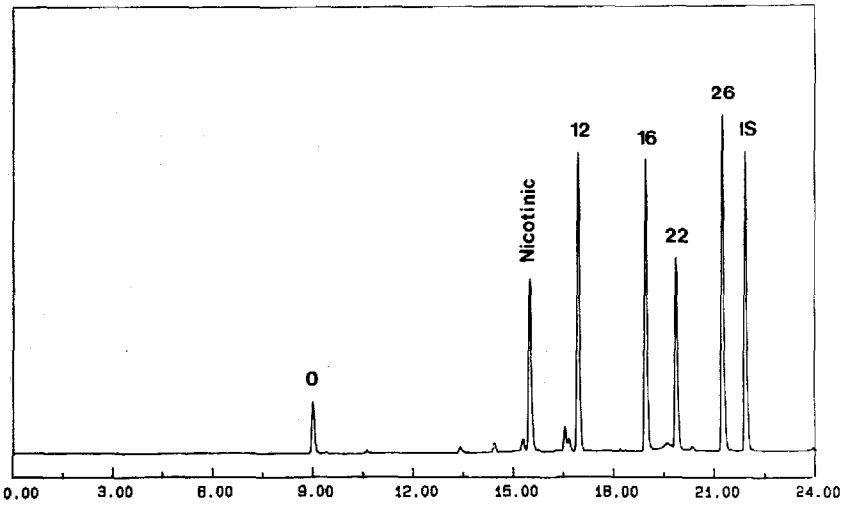

Fig. 11. Separation of the PNB derivatives of standard mixture III. The sample was not exposed to Dowex 50W-X8 in order not to remove nicotinic acid. For the same reason, the clean-up procedure A (filtration through Sep-Pak silica) was used.

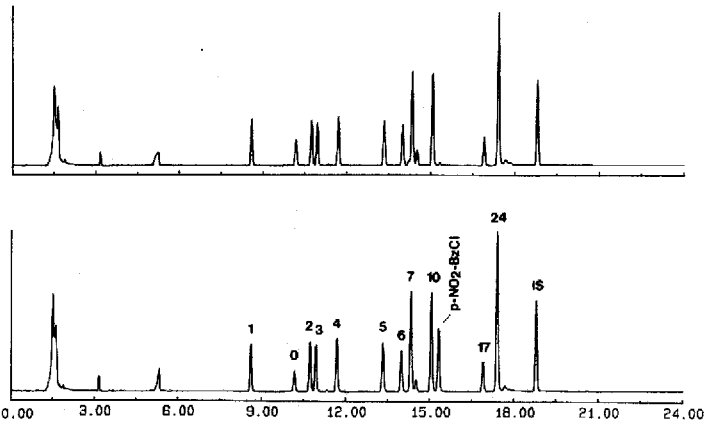

Fig. 12. Result of the removal of added chloride ions by treatment with silver tetrafluoroborate. Lower chromatogram, standard mixture I spiked with a 1.1 *M* equivalent of sodium chloride. Upper chromatogram, same spiked mixture that was exposed to an excess of silver ions (2 equivalents) and derivatized as described after filtration.

numbers) are missing. It is of interest that the three missing acids, phosphoric, malic and citric acids, have strong chelating properties. The same observation was made elsewhere for tartaric acid, the chelating behaviour being similar.

The Dowex treatment (whether before or after the derivatization) also removed any compound with basic sites. Besides caffeine, for instance, it eliminated pyridine-3-carboxylic acid (nicotinic acid, niacin) which otherwise gave the corresponding PNB ester in a good relative yield (Fig. 11). Nicotinic acid PNB ester eluted after 15.5 min (see also Table I). However, because the analysis of this compound in coffee implied a particular procedure, its determination was not investigated further in the present study.

Removal of interference due to the presence of chloride ions

Samples containing chloride ions gave rise to the formation of PNB chloride upon derivatization with PNBDI. This compound interfered with propionic acid PNB ester in the chromatographic separation, having the same retention time (Table I)*. On a simple exposure to silver ions, the chlorides could be cleanly removed without perturbing the compounds of interest, as can be seen in Fig. 12. The lower trace corresponds to the separation, after derivatization, of the standard mixture I to which was added a 1.1 *M* equivalent (based on each acid) of sodium chloride. The upper trace shows the same spiked mixture, which was primarily treated with an excess of aqueous silver tetrafluoroborate (unlike nitrate, for instance, this counter-ion did not react with PNBDI). The expected precipitate was filtered off and the remaining silver cations were removed during the usual pre-treatment of the sample with the cation-exchange resin.

* In the context of the HPLC separation of short-chain carboxylic acid *p*-bromophenacyl esters, the same interference between chloride and propanoate ions was noticed[14].

TABLE II

RESULTS OF THE DETERMINATION OF THE MAIN CARBOXYLIC ACIDS AND PHOSPHORIC ACID IN A SAMPLE OF INSTANT COFFEE SPIKED WITH VARIOUS QUANTITIES OF ACIDS AND CALCULATION OF THE RECOVERY LEVELS

Each value represents the mean of four determinations.

Acid	*Added amount (μmol/ml)*	*Found*	*R.S.D. (%)*	*Recovery level (%)*	*Content (g/kg)*
Quinic	–	2.38	4.0	–	22.85
	1	3.38	4.8	100.2	
	2	4.45	1.4	101.6	
	4	5.96	5.4	93.5	
Glycolic	–	1.01	1.7	–	3.85
	0.4	1.41	1.5	99.5	
	0.8	1.78	0.7	98.3	
	1.6	2.57	1.9	98.4	
Pyroglutamic	–	0.46	3.0	–	3.00
	0.4	0.95	6.4	110.2	
	0.8	1.34	4.9	105.8	
	1.6	2.22	2.2	107.6	
Lactic	–	0.44	1.1	–	2.00
	0.4	0.82	2.0	97.2	
	0.8	1.21	0.4	97.6	
	1.6	2.00	0.2	98.0	
Formic	–	2.70	1.6	–	6.21
	1	3.68	2.0	99.4	
	2	4.55	1.2	96.9	
	4	6.52	0.7	97.3	
Acetic	–	1.47	1.9	–	4.45
	1	2.46	0.4	99.5	
	2	3.39	0.3	97.7	
	4	5.31	0.3	97.1	
Malic	–	1.31	1.1	–	8.75
	0.4	1.74	0.2	101.9	
	0.8	2.12	0.2	100.5	
	1.6	2.90	0.3	99.7	
Phosphoric	–	1.28	6.7	–	6.27
	0.4	1.76	5.0	104.9	
	0.8	2.16	3.9	103.6	
	1.6	3.07	3.1	106.7	
Citric	–	2.15	0.2	–	20.61
	1	3.23	0.2	102.6	
	2	4.17	0.1	100.6	
	4	6.18	0.3	100.5	

TABLE III

CARBOXYLIC ACID CONTENT OF APPLE AND ORANGE JUICES AND OF WHITE AND RED WINES AS DETERMINED BY THE PRESENT METHOD

Acid	*Apple juice (g/l)*	*Orange juice (g/l)*	*White wine (g/l)*	*Red wine (g/l)*
Quinic	0.60	–	–	–
Lactic	–	–	2.43	1.69
Acetic	–	–	0.20	0.39
Tartaric	–	–	1.56	1.62
Malic	6.87	1.11	0.06	0.15
Succinic	–	–	0.42	0.58
Citric	0.25	8.47	0.16	0.10

Application examples

Coffee samples. Eight major aliphatic acids and phosphoric acid were quantitated in coffee samples (see Fig. 10) by the internal standard method (integration of peak areas). The results are reported in Table II. Mixture I was used as the calibration standard. The precision of the determination was generally very good, with relative standard deviations (R.S.D.) < 2% for all of the standard acids. The accuracy of the analysis in coffee samples was also satisfactory, as judged by the recovery rates (94–108%) for each component and the R.S.D. values which are listed in the table.

Several peaks were unknown impurities, while others corresponded to minor carboxylic acids tentatively identified by their retention times. Thus, the following compounds were detected: furan-2-carboxylic (12), malonic (14), itaconic (19), fumaric (25) and mesaconic (26) acids. Their quantitation, however, necessitates the

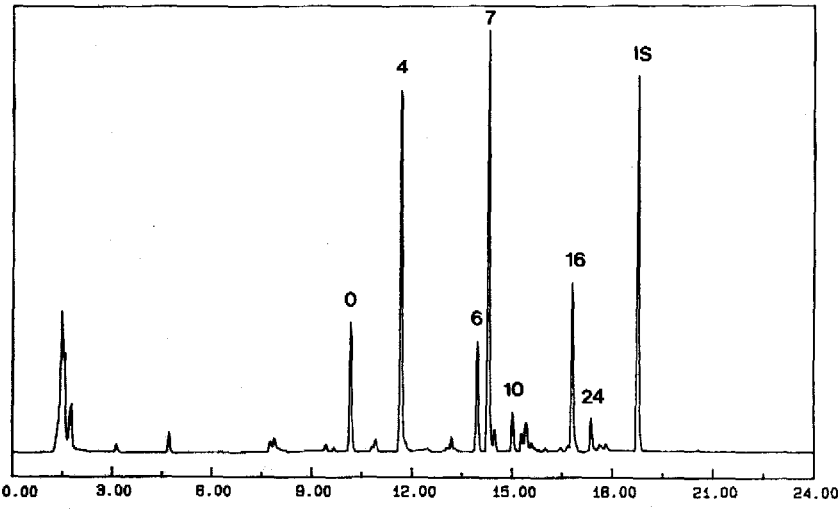

Fig. 13. Profile of the carboxylic acid PNB esters of a sample of French red wine. The analytical conditions were the same as in Fig. 4.

development of a particular chromatographic system, and lies outside the objective of this study.

Wine and fruit juices. Two samples of wine (a Swiss white wine and a French red wine) and samples of bottled orange and apple juice were analysed. The same derivatization scheme was applied with clean-up procedure B. The results of the determinations are reported in Table III. These values compare very well with those of the literature[10,23,34,35].

To illustrate this result, the separation of the carboxylic acid PNB esters of a sample of red wine is shown in Fig. 13. Nearly all peaks can be identified and they are practically all baseline resolved. This last example clearly shows both the ease and the high precision with which the identification and quantitation of these acids could be achieved.

CONCLUSION

We feel that the method reported here will be a good alternative to other HPLC methods based either on ion-exchange or reversed-phase systems (without or with derivatization). It does not necessitate long and tedious clean-up, extraction or isolation operations. The crude samples are primarily treated with a strong cation-exchange resin to liberate the free carboxylic acids, derivatized as described and cleaned up with the same ion-exchange resin to remove the excess of reagent. All the operations are conducted in the same vial, which is used in the automated sample injector, allowing many samples to be processed at the same time. Two internal standards may be used according to their solubility properties: cyclohexanecarboxylic acid for liposoluble material and benzylmalonic acid for aqueous samples. The procedure is rapid, simple and economical as it avoids the use of expensive disposable sample clean-up cartridges (Sep-Pak or equivalent). Moreover, it has the advantage of allowing the determination of quinic acid, the PNB ester of which was totally removed in the former procedure. At the same time, a better recovery of the PNB esters of pyroglutamic and tartaric acid was also obtained. Using dioxane as the solvent allows the simultaneous determination of phosphoric acid.

The procedure described here appears to be of general scope, though oxalic, pyruvic and probably also other α-oxo-carboxylic acids did not give the desired derivative under the conditions used (a previous protection of the keto acids by oxime formation prior to the reaction with PNBDI is worth investigating). The application of the method to the determination of long-chain fatty acids and bile acids should be considered.

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REFERENCES

1 R. Schwarzenbach, *J. Chromatogr.*, 251 (1982) 339.
2 R. Schwarzenbach, *Mitt. Geb. Lebensm. Hyg.*, 75 (1984) 51.
3 B. S. Buslig, C. W. Wilson and P. E. Shaw, *J. Agric. Food Chem.*, 30 (1982) 342.
4 C. W. Wilson, P. E. Shaw and C. W. Campbell, *J. Sci. Food Agric.*, 33 (1982) 777.

5 R. Anderson and B. Hedlund, *Z. Lebensm. Unters. Forsch.*, 176 (1983) 440.
6 R. J. Bushway, J. L. Bureau and D. F. McGann, *J. Food Sci.*, 49 (1984) 75.
7 S. H. Ashoor and J. Welty, *J. Chromatogr.*, 287 (1984) 452.
8 S. H. Ashoor and M. J. Knox, *J. Chromatogr.*, 299 (1984) 288.
9 W. Böhme, U. Oehme and M. Steinwand, *Lebensmittelchem. Gerichtl. Chem.*, 38 (1984) 88.
10 E. Mentasti, M. C. Gennaro, C. Sarzanini, C. Baiocchi and M. Savigliano, *J. Chromatogr.*, 322 (1985) 177.
11 P. Pfeiffer and F. Radler, *Z. Lebensm. Unters. Forsch.*, 181 (1985) 24.
12 C. Baiocchi, E. Campi, M. C. Gennaro, E. Mentasti and C. Sarzanini, *Ann. Chim. (Rome)*, 73 (1984) 37.
13 R. L. Patience and J. D. Thomas, *J. Chromatogr.*, 234 (1982) 225.
14 R. L. Patience and J. D. Thomas, *J. Chromatogr.*, 249 (1982) 183.
15 H. Ménard, A. Gaboury, D. Bélanger and C. Roy, *J. Anal. Appl. Pyrol.*, 6 (1984) 45.
16 S. T. Ingalls, P. E. Minkler, C. L. Hoppel and J. E. Nordlander, *J. Chromatogr.*, 299 (1984) 365.
17 E. Vioque, M. P. Maza and F. Millán, *J. Chromatogr.*, 331 (1985) 187.
18 M. J. Cooper and M. W. Anders, *Anal. Chem.*, 46 (1974) 1849.
19 D. Knapp and S. Krueger, *Anal. Lett.*, 8 (1975) 603.
20 E. Grushka, H. Durst and E. Kikta, Jr., *J. Chromatogr.*, 112 (1975) 673.
21 B. Shaikh, N. Pontzer, J. Molina and M. Kelsey, *Anal. Biochem.*, 85 (1978) 47.
22 E. Heftmann, G. Saunders and W. Haddon, *J. Chromatogr.*, 156 (1978) 71.
23 W. Steiner, E. Müller, D. Fröhlich and R. Battaglia, *Mitt. Geb. Lebensm. Hyg.*, 75 (1984) 37.
24 Z. L. Bandi and E. S. Reynolds, *J. Chromatogr.*, 329 (1985) 57.
25 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, *J. Chromatogr.*, 234 (1982) 121.
26 W. Elbert, S. Breitenbach, A. Heftel and J. Hahn, *J. Chromatogr.*, 328 (1985) 111.
27 R. Farinotti, P. Siard, J. Bourson, S. Kirkiacharian, B. Valeur and G. Mahuzier, *J. Chromatogr.*, 269 (1983) 81.
28 H. Lingeman, A. Hulshoff, W. J. M. Underberg and F. B. J. M. Offermann, *J. Chromatogr.*, 290 (1984) 215.
29 W. D. Korte, *J. Chromatogr.*, 243 (1982) 153.
30 U. Engelhardt and H. G. Maier, *Fresenius' Z. Anal. Chem.*, 320 (1985) 169.
31 U. Engelhardt and H. G. Maier, *Z. Lebensm. Unters. Forsch.*, 181 (1985) 206.
32 E. Schmidt, E. Daebritz and K. Thulke, *Ann. Chem.*, 685 (1965) 161.
33 V. M. Clark, J. J. Michalski and A. R. Todd, *J. Chem. Soc.*, (1949) 815.
34 C. Droz and H. Tanner, *Schweiz. Z. Obst. Weinbau*, 15 (1982) 434.
35 L. Lin and H. Tanner, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 126.