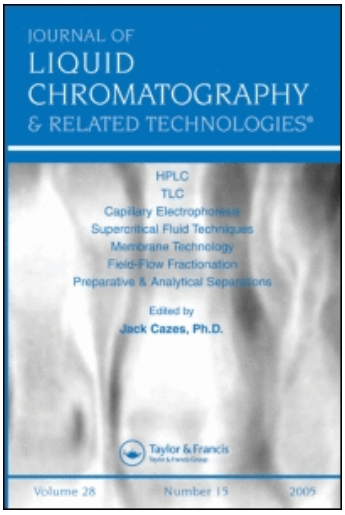

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Sara C. Cunha[a]; Isabel M. P. L. V. O. Ferreira[a]; José O. Fernandes[a]; Miguel A. Faria[a]; M. Beatriz[a]; P. P. Oliveira[a]; M. A. Ferreira[a]
[a] Universidade do Porto, Porto, Portugal

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Sara C. Cunha, Isabel M. P. L.V. O. Ferreira,*
José O. Fernandes, Miguel A. Faria, M. Beatriz,
P. P. Oliveira, and M. A. Ferreira

CEQUP/Faculdade de Farmácia, Universidade do Porto,
Serviço de Bromatologia, R. Aníbal Cunha, 164,
4050-047, Porto, Portugal

ABSTRACT

This paper describes a procedure performed by high performance liquid chromatography/UV detection for quantification of the major carboxylic acids in table olives (lactic, acetic, succinic, and citric acids); derivatization of carboxylic acids with O-(4-nitrobenzil)-N,N'diisopropylisourea (PBNDI) was performed.

The sample preparation involved deproteination with ethanol and the use of strong cation-exchange resin (Dowex 50W-X8) to liberate the free carboxylic acids. The same resin was used to remove the excess of derivatizing reagent. The chromatographic separation was achieved using reverse-phase column C18 (ODS). The mobile phase used was a gradient of water and acetonitrile at a flow-rate of 1 mL/min. The effluent was monitored using a UV detector at 265 nm.

*Corresponding author.

The method allowed for well-resolved peaks of lactic, acetic, succinic, and citric acids in less than 25 min. Precision and recovery assays were performed with good results for the acids under study.

Nineteen samples of table olives available on the Portuguese market, including nine samples of green olives and 10 samples of black olives, were successfully monitored applying this methodology. The concentrations of carboxylic acids are expressed in percentage of moist olive pulp and ranged from not detected to 477.3 mg of lactic acid/100 g of moist olive pulp and 9.43 to 232.1 mg of acetic acid/100 g of moist olive pulp. Citric acid was detected only in two samples of green table olives with concentrations of 24.7 and 188.3 mg/100g of moist olive pulp. Succinic acid was detected in five samples of green table olives ranging from 10.1 to 25.8 mg/100 g moist olive pulp, and in two samples of black table olives with concentrations of 10.7 and 29.4 mg/100 g of moist olive pulp.

INTRODUCTION

Carboxylic acids are important constituents of table olives, representing about 1.5% of the weight of the pulp of the final product.[1] They are of great importance, not only because they influence flavour, stability, and keeping quality of the product, but also owing to their buffering capacity during the fermentation stage and further storage.[1,2] Carboxylic acids present metabolic activity and are intermediate products of formation and degradation of other compounds. For those reasons, the quantitative determination of carboxylic acids in table olives is required for quality control purposes, meeting various laws and regulations, and for labelling requirements.

The analysis of carboxylic acids in table olives and brine include gas chromatographic methods,[3] and also high performance liquid chromatography (HPLC).[4,5] Conversion of the carboxylic acids to the corresponding ester derivatives enhances their detectability by UV detection, because the derivatized carboxylic acids have their maximum absorptivity around 265 nm, and this zone offers better selectivity than the 210 nm used for the direct method.[6] Several derivatising reagents, e.g. phenacyl,[7,8] naphthacyl,[9] *p*-nitrophenyl, and *p*-nitrobenzyl[6] were proposed for the determination of carboxylic acids in various samples, with satisfactory results.

This paper describes an HPLC method for quantification of the major carboxylic acids in table olives (lactic, acetic, succinic, and citric acids, LA, AA, SA, CA, respectively). This matrix exhibits a complex composition, thus justify-

ing our first objective: optimization of the extraction procedure to free these compounds from the olive pulp, followed by derivatization with O-(4-nitrobenzil)-N,N`diisopropylisourea (PBNDI).[6] Furthermore, another objective of this research work was to demonstrate the applicability of the method via recovery and precision studies. The final objective was to apply the developed method to the determination of the quantitative profiles of carboxylic acids in table olives available on the Portuguese retail market.

EXPERIMENTAL

Apparatus

The chromatographic analysis was carried out in a Gilson, high performance liquid chromatograph (Gilson Medical Electronics, Villiers le Bel, France), equipped with a type 305 pump, a type 306 pump and a type 7125 Rheodyne Injector (Rhedodyne, Cotati, CA, U.S.A.) with a 10 μL loop. A Gilson 118, variable long wave ultra violet detector was also used.

The chromatographic separation was achieved with a Hypersil ODS (3 μm, 250 x 4.6 mm). The column was preceded by a pre-column C_{18} (Nucleosil; 30 x 4 mm). The integrator used was a Varian (Varian, Harbor City, CA, U.S.A.) model 4290.

Reagents and Standards

Aliphatic carboxylic acids (or their sodium salts) were purchased from Fluka (Buchs, Switzerland) and Aldrich (Steinheim, Germany) Chemicals Company. The strong cation-exchange resin Dowex-50W-X8 (100-200 mesh; p.a.) was obtained from Fluka and was activated with methanol, water, 0.1 M hydrochloric acid, and water. The derivatizing reagent, O-(4-nitrobenzil)-N,N'diisopropylisourea (PBNDI) purified, was obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, methanol, ethanol n-hexane, and dioxane were purchased from Merck (Darmstad, Germany). All the reagents were analytical grade.

Sampling

Nineteen samples were assayed, which included 9 green table olives and 10 black olives. They were randomly purchased on the market.

Sample Preparation

2.5 g of homogenised sample was thoroughly mixed with 10 mL of ethanol. The extract was heated in a water bath for 60 min. at 60°C. After cooling, 2 mL of n-hexane were added and the resulting mixture was centrifuged at 5000 rpm for 10 min. The supernatant was filtrated, and treated for 15 minutes with 0.5 g of strong cation-exchange resin (Dowex-50W-X8) previously activated; the mixture was briefly shaken. A portion of the clear supernatant was used for the subsequent derivatization.

Derivatization Procedure

The sample (or standard) solutions (50 μL) were placed in 3.5-mL PTFE-line screw-capped amber vials. The carboxylic acids were then derivatized with a mixture of 20 mg of PNBDI in 500 μL of dioxane, by heating in a thermostatic block for 60 min. at 80°C. After cooling, the solution was diluted by the addition of 1 mL of acetonitrile and 0.5 g (50 mg/mg PNBDI) of Dowex 50W-X8 were 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 0.22 μm disposable LC filter disk.

Chromatography

The eluent used was a gradient of water (A) and acetonitrile (B). Elution was performed at a solvent flow rate of 1 mL/min with linear gradients as follows: 0.30% B, 10.45% B, 20.55% B, 35.80% B, keeping these conditions during 3 min and returning to the initial conditions within 2 min. Analyses were conducted at ambient temperature. Detection was accomplished with UV detection, and chromatograms were recorded at 265 nm.

LA, AA, SA, and CA in samples were identified by chromatographic comparison of the retention time of standards. Quantification was based on the external standard method.

Statistical Analysis

Data are presented as the mean ± standard deviation. The results were statistically analysed by analysis of variance (ANOVA) followed by Fisher's PLSD test. Differences were considered significant for $p<0.05$. Statistical analyses were carried out with the Statview TM 4.0 statistical package (Abacus concepts, Berkeley, CA, USA).

RESULTS

Analytical Curve and Detection Limit

Under the assay conditions described, a linear relationship between the concentration of LA, AA, SA, CA, and UV absorbance at 265 nm was obtained. This linearity was maintained over the concentration range of 0.4 - 4 g/L for LA and AA, and of 0.2 - 2 g/L for SA and CA.

The detection limits calculated as the concentration corresponding to three times the background noise, was 0.056 g/L for LA, 0.041g/L for AA, 0.046 g/L for SA, and 0.098 g/L CA.

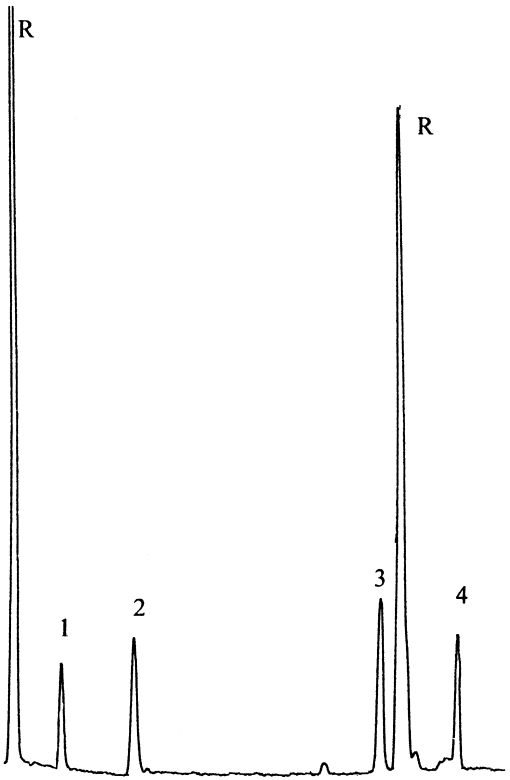

Figure 1. Typical chromatogram for separation of four carboxylic acids (chromatographic conditions described in the text): 1 – LA (RT 7.21), 2 – AA (RT 10.35), 3 – SA (RT 23.03), 4 – CA (RT 25.90). R – Peaks from derivatising reagent. The concentrations of the acids injected onto the column were 5.00 mg LA/mL, 1.00 mg AA/mL, 0.50 mg SA, and CA/mL.

Figure 1 shows a typical chromatogram for separation of the four carboxylic acids.

Validity of the Method

Four different extractions, as described above, were made for samples 2 and 16, and were subsequently injected in duplicate, to evaluate the reproducibility of the method. The standard deviation and CV are listed in Table 1.

Recovery studies were performed on another two randomly chosen samples (8 and 12), to verify the effectiveness of the extraction step and the accuracy of the purposed methodology. Table 2 presents the results obtained from the recovery studies.

Chromatograms and Results for Table Olive Samples

Tables 3 and 4 present the concentration of LA, AA, SA, and CA assayed in green and black table olives, respectively.

As apparent from Tables 3 and 4, green and black table olives provided different quantitative profiles of carboxylic acids. The prevailing carboxylic acid in both types of table olives were LA and AA, but their concentrations were affected differently by the green and black type. Analysis of variance showed that this variable (olive type) was not significant for AA ($p=0.9289$), in contrast to the LA content, which was significantly affected ($p=0.0001$); black table olives presented lower concentrations of LA (Tables 3 and 4).

The data pertaining to the LA evaluation indicate significant differences ($p=0.0001$) among the samples of green table olives, which is confirmed by the F-value obtained from the statistical analysis ($F= 31.07$). Among the assayed samples, 56% contained levels of LA around 400 mg/100 g of moist olive pulp

Table 1. Results of Standard Deviation and CV Found in the Reproducibility Assays (n=8)

Samples	Green Olives			Black Olives		
Acids	Mean mg/100g	S.D. mg/100g	CV %	Mean mg/100g	S.D. mg/100g	CV %
Lactic	360.17	0.25	5.87	94.26	0.05	4.9
Acetic	87.65	0.01	1.87	125.66	0.01	1.5
Citric	188.30	0.06	3.36	n.d.	n.d.	n.d.

Table 2. Results for the Recoveries Obtained by the Standard Additions Method

Samples		Green Olives		Black Olives	
Carboxylic acids	Added mg/100g	Found[a] mg/100g	Recovery %	Found[a] mg/100g	Recovery %
Lactic	0.00	477.3		56.9	
	1.5		110.8		95.6
	3.0		108.4		103.2
	4.5		104.6		110.4
	6		101.6		98.2
Acetic	0.00	102.4		34.8	
	1.0		101.5		107.4
	2.0		111.7		98.5
	3.0		105.6		102.9
	4.0		102.4		98.7
Succinic	0.00	10.1		n.d.	
	1.0		89.2		96.6
	2.0		99.9		91.3
	3.0		101.4		102.0
	4.0		107.2		98.7
Citric	0.00	n.d.		n.d.	
	1.0		92.8		93.4
	2.0		109.5		90.8
	3.0		95.2		95.5
	4.0		111.2		107.2

[a] Mean value for 2 assays for each studied concentration.

(samples 4, 5, 6, 7, 9). Sample 3 was the only one with a concentration, which seriously deviated from the mean value.

Greater variability among samples of green table olives was noticed for AA (as apparent from the F value, F=84.6), with concentrations varying between 9.43 and 221.7 mg/100 g of moist olive pulp (Table 3). However, 67% of the analysed samples registered AA contents within the range of 87.7 to 129.7 mg/100g of moist olive pulp.

In contrast, a large variability among black table olives for LA (F=243.8, p=0.0001) was reported. Concentrations varied between not detected and 399.1 mg/100g of moist olive pulp. Significant statistical similarities were found between samples 10 and 14, 11 and 16, 12 and 19. LA was not in samples 17 and 18, which indicate that lactic acid fermentation did not occur in those samples. With respect to the content of AA, the dispersion among samples was lower (F=69.35, p=0.0001), 50% of the samples contained levels of AA around

Table 3. Results Obtained in the Monitoring of Carboxylic Acids in Green Table Olives*

Samples	Lactic	Acetic	Succinic	Citric
1	302.2±15.1 [a]	158.0±8.5 [a]	25.8±1.3	24.7±1.5
2	360.2±11.4 [b]	87.7±0.2 [b]	n.d.	188.3±3.0
3	214.1±9.1 [c]	221.7±12.0 [c]	23.0±1.0	n.d.
4	412.4±20.9 [d,e]	129.7±1.3 [d]	16.7±0.67	n.d.
5	420.2±14.7 [d,e]	94.2±4.9 [b,e]	n.d.	n.d.
6	437.2±18.1 [d,f]	109.7±6.9 [e,f]	n.d.	n.d.
7	391.1±3.0 [b,e]	123.6±6.1 [d,f]	n.d.	n.d.
8	477.3±16.6 [f]	102.4±4.4 [b,e]	10.1±0.7	n.d.
9	389.3±9.6 [b,e]	9.43±0.40 [g]	16.5±0.4	n.d.

*Values are expressed as mean ± standard deviation of two determinations (mg of carboxylic acid/100 g of moist olive pulp). a,b,c,d,e,f,g - means in columns without common superscripts are significantly different ($p<0.05$); n=2.

90 mg/100 g of moist olive pulp (samples 10, 11, 14, 15 and 17), as shown in Table 4.

Succinic acid was detected in five samples of green table olives ranging from 10.1 to 25.8 mg/100 g moist olive pulp, and in two samples of black table olives with concentrations of 10.7 and 29.4 mg/100g of moist olive pulp. Citric acid was detected only in two samples of green table olives with concentrations of 24.7 and 188.3 mg/100g of moist olive pulp.

Table 4. Results Obtained in the Monitoring of Carboxylic Acids in Black Table Olives

Samples	Lactic mg/100g	Acetic mg/100g	Succinic mg/100g	Citric mg/100g
10	330.2±13.6 [a]	93.5±4.2 [a,d]	n.d.	n.d.
11	123.2±3.3 [b]	94.0±5.2 [a,d]	n.d.	n.d.
12	56.9±1.4 [c]	34.8±1.2 [b]	n.d.	n.d.
13	191.4±10.0 [d]	232.1±12.9 [c]	29.4±1.3	n.d.
14	306.6±18.3 [a]	77.6±4.1 [a]	10.7±0.6	n.d.
15	399.1±14.4 [e]	98.8±2.4 [d]	n.d.	n.d.
16	94.3±0.4 [b]	125.7±5.5 [e]	n.d.	n.d.
17	n.d. [f]	95.9±9.38 [a,d]	n.d.	n.d.
18	n.d. [f]	150.1±8.0 [f]	n.d.	n.d.
19	61.8±0.3 [c]	164.8±2.8 [f]	n.d.	n.d.

*Values are expressed as mean ± standard deviation of two determinations (mg of carboxylic acid/100 g of moist olive pulp). a,b,c,d,e,f, - means in columns without common superscripts are significantly different ($p<0.05$); n=2.

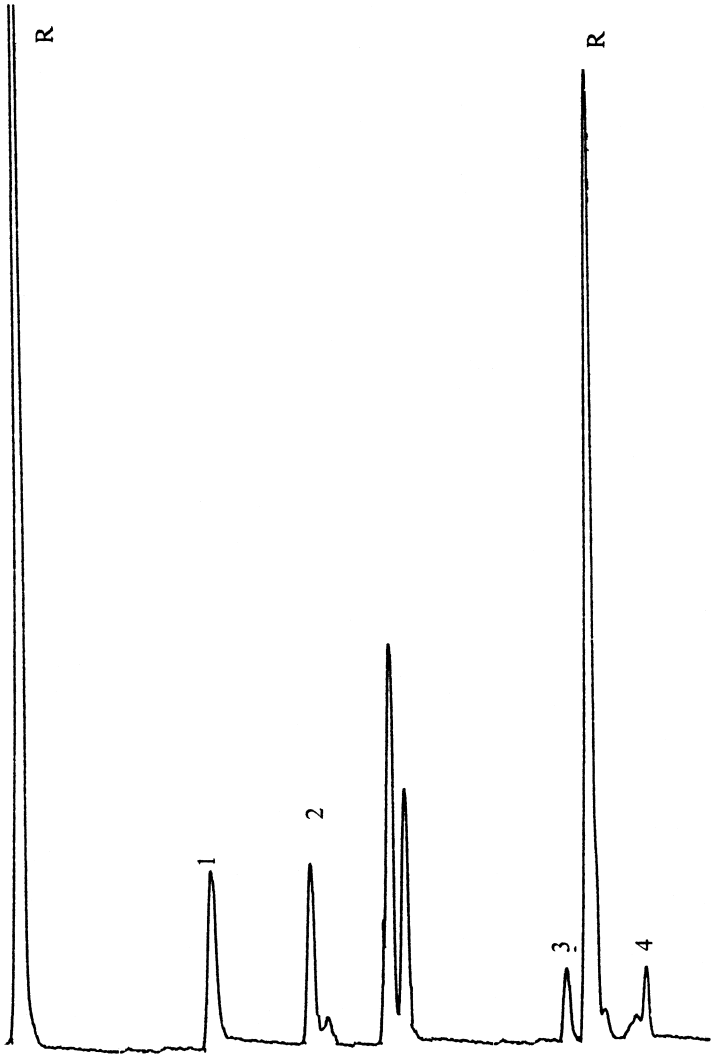

Figure 2. Typical chromatogram for a green table olive sample which presented the carboxylic acids. The numbers correspond to the numbers in Fig. 1 with respect to peak identification.

Figure 2 shows the typical chromatogram for green table olive which presented the carboxylic acids lactic acid, acetic acid, succinic acid, and citric acid. The concentrations of LA, AA, SA was 474.5 mg/100 g of moist olive pulp, 111.7 mg/100 g of moist olive pulp, and 9.3 mg/100 g of moist olive pulp, respectively; CA was present in trace amounts. Table olives are a complex matrix

and other compounds, namely chlorides, are extracted together with carboxylic acids, so some unidentified peaks appeared on the chromatograms, but their presence did not interfere with the evaluation of the carboxylic acids assayed.

CONCLUSIONS

Considering the complexity of the analysis, which included extraction of carboxylic acids from olive matrix, derivatization, and chromatographic determination, the results obtained for the validation of the method must be considered satisfactory. The chromatographic gradient was optimised to achieve chromatograms with no interfering components on the retention times of the carboxylic acids under study.

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