Microwave-assisted digestion procedures for biological samples with diluted nitric acid: Identification of reaction products

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ABSTRACT

Microwave-assisted sample preparation using diluted nitric acid solutions is an alternative procedure for digesting organic samples. The efficiency of this procedure depends on the chemical properties of the samples and in this work it was evaluated by the determination of crude protein amount, fat and original carbon. Soybeans grains, bovine blood, bovine muscle and bovine viscera were digested in a cavity-microwave oven using oxidant mixtures in different acid concentrations. The digestion efficiency was evaluated based on the determination of residual carbon content and element recoveries using inductively coupled plasma optical emission spectrometry (ICP OES). In order to determine the main residual organic compounds, the digests were characterized by nuclear magnetic resonance (^{1}H NMR). Subsequently, studies concerning separation of nitrobenzoic acid isomers were performed by ion pair reversed phase liquid chromatography using a C18 stationary phase, water:acetonitrile:methanol (75:20:5, v/v/v) + 0.05% (v/v) TFA as mobile phase and ultraviolet detection at 254 nm. Sample preparation based on diluted acids proved to be feasible and a recommendable alternative for organic sample digestion, reducing both the reagent volumes and the variability of the residues as a result of the process of decomposition. It was shown that biological matrices containing amino acids, proteins and lipids in their composition produced nitrobenzoic acid isomers and other organic compounds after cleavage of chemical bonds.

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1. Introduction

Sample preparation is one of the most time-consuming steps and also one of the main sources of contamination in an analytical procedure. This step must become easier, cheaper and faster according to green chemistry trends. Spectrochemical instrumental techniques for elemental analysis generally require complete sample decomposition. The destruction of organic matter in biological samples is the main parameter when decomposition efficiency is evaluated [1]. However, this efficiency depends on the original chemical composition of the sample and the temperature used during the digestion process. For instance, when concentrated nitric acid is employed carbohydrates matrices are rapidly decomposed at 140 °C, while protein molecules are decomposed at 150 °C, and lipid molecules require approximately 160 °C [2]. No correlation was observed between residual carbon content (RCC) and protein content when fat and protein contents of biological samples were correlated with RCC after microwave-assisted digestion. However, it was shown that protein-rich samples are easily decomposed than fat rich materials [3].

Nitric acid is frequently used in sample decomposition processes due to its simple manipulation, easy purification and efficiency in oxidation of organic compounds present in biological samples [4,5]. Concentrated nitric acid suffers thermal decomposition producing different water soluble oxides, such as NO, NO_2, NO_3^-, N_2O, N_2O_3, N_2O_4, N_2O_5, N_2O_6, HNO and HNO_2, with oxidant characteristics [6]. The formation of these gaseous decomposition products caused a sharp increase in pressure with barely no temperature change.

Closed-vessel microwave-assisted digestion with diluted acid solutions is an efficient alternative for sample preparation of organic samples for inorganic analysis using spectrochemical techniques [7–10]. Compared to concentrated nitric acid, diluted nitric acid solutions generate less residues, led to lower standard deviations and do not require high dilution factors before analyte measurements. Additionally, it is also necessary to take into account the involved chemical processes. Gaseous NO is generated during the oxidation of the organic constituents by HNO_3 action. Nitrous oxide is volatilized from the heated solution and reacts with O_2

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present in the gas phase of the reaction vessel. Consequently, NO_2 is generated and this gas is reabsorbed in the solution. Then, a disproportionate reaction occurs leading to the formation of $NO_3{}^-$ and NO and the reaction cycle repeats itself up to the point that there is no O_2 in the gas phase of the closed vessel [11].

Previous studies were developed to identify organic residues generated during acid digestion. Mörner carried out systematic studies involving decomposition of protein samples by conventional heating with HNO_3 [12]. Benzoic, oxalic, picric, terephthalic acids and *m*- and *p*-nitrobenzoic acids (NBAs) were the products identified after decomposition. Species of *p*-nitrophenylalanine were described by Takayama and Tsubuku as mild nitration products in protein samples [13].

Pratt et al. identified organic residues of decomposition of bovine liver (National Institute of Science and Technology, standard reference material 1577a) using high performance liquid chromatography (HPLC) and voltammetric techniques. The main decomposition products formed were isomers of *m*- and *p*-nitrobenzoic acids when applying microwave-assisted nitric acid digestion [14].

Reaction products formed during the digestion of biological samples were also investigated by other authors [15–18]. Results have shown the need of employing different sample digestion methods according to the sample matrix and the technique being used [16]. The main digestion products were isomeric mixtures of nitrobenzoic acids and aromatic compounds [15,17,18].

Residual carbon contents were employed in order to evaluate the efficiency of microwave-assisted acid digestion of plant materials by using different concentrations of nitric acid with hydrogen peroxide. It was demonstrated by characterization of products formed by ^1H NMR that diluted acid nitric solutions led to less complex mixtures of residues when compared to concentrated nitric acid [19].

In the work here described the efficiency of microwave-assisted acid digested procedures for animal and plant samples were evaluated by identifying the reaction products using spectroscopic and chromatographic techniques.

2. Experimental

2.1. Instrumentation and instrument parameters

A model 6750 Freezer/Mill Spex Certiprep (Metuchen, USA) with a self-contained liquid nitrogen bath was used with model 6751 grinding vials.

Total carbon content was determined by elemental analyzer CHNS EA 1108, Fisons Instruments (Italy). Kjeltec Auto Sampler System 10035, Tecator (Vancouver, Canada) was used for crude protein determination. The fat content was determined using an accelerated solvent extractor—ASE, Ankom, model XT 2016 (Macedon, USA).

A microwave oven system equipped with perfluoalcoxi (PFA) vessels and temperature sensor Multiwave, Anton-Paar (Graz, Austria) was employed for sample digestion.

Residual carbon and mineral contents were determined by inductively coupled plasma optical emission spectrometry ICP OES Vista RL, Varian, Mulgrave (Australia).

The ^1H NMR spectra were acquired using a Varian Inova 400 spectrometer. The ^1H NMR spectra were recorded in deuterated methanol at 30 °C and tetramethylsilane (TMS) was added as an internal standard, using 32 transients, a $\pi/2$ pulse of 10 μs and a delay of 2 s and an acquisition time of 3.7 s.

The high performance liquid chromatographic system (HPLC) consisted of a Shimadzu LC-10ATVP pump (Kyoto, Japan), with the pump containing a valve FCV-10AL for selecting solvent, an autoinjector model SIL 10AVP, a degasser model DGU-14A, a SCL 10AVP

interface and a SPD-6AV UV-visible detector operated at 254 nm. Data were acquired by Shimadzu Class-VP software.

Separation of *o*-, *m*- and *p*-nitrobenzoic isomers was achieved using a C18 column (250 mm × 4.6 mm I.D., 5 μm particle size), purchased from Thermo Electron Corporation (Waltham, USA). Chromatographic experiments were carried out using an isocratic elution mode with a mobile phase consisting of water:acetonitrile:methanol (75:20:5, v/v/v) + 0.05% (v/v) trifluoracetic acid (TFA) at a flow rate of 1.0 mL min^{-1}. The column was kept at room temperature and the volume of injection was 10 μL. *o*-, *m*- and *p*-nitrobenzoic acids were identified based on the retention time of standard solutions for each compound.

2.2. Reagents, standards and samples

Deionized water with a Milli-Q® System Millipore (Bedford, MA, USA) was used to prepare all solutions. All glassware was immersed in 10% (v/v) HNO_3 for 36 h and rinsed with water.

Reagent grade concentrated nitric acid (Carlo Erba, Italy) and 30% (w/v) hydrogen peroxide (Mallinckrodt, Mexico) were employed.

Multielement reference solutions were prepared from 1000 mg L^{-1} stock solutions Titrisol® Merck (Darmstadt, Germany). Carbon reference solutions were prepared from 1000 mg L^{-1} urea sock solution Synth (São Paulo, Brazil).

HPLC grade methanol, acetonitrile and chloroform were purchased from J.T. Baker (Phillipsburg, NJ, USA). Analytical grade ethyl ether was also provided by J.T. Baker.

Bovine blood, bovine viscera and soybeans grains samples were all provided by Embrapa Pecuária Sudeste (São Carlos, SP, Brazil). Certified bovine muscle NIST SRM 8414 was used for checking accuracy and method validation (Gaithersburg, MD, USA).

The standard stock solutions for *o*-, *m*- and *p*-nitrobenzoic acid isomers were purchased from Across Organics (Geel, Belgium).

2.3. Procedure

2.3.1. Sample preparation for ICP OES determination

All samples were ground in a freezer mill operated at liquid nitrogen temperature (−195 °C). Before grinding, these samples were lyophilized in glass flasks connected to the vacuum system of a freeze-drier operated at −195 °C for 80 h, and further stored at −10 °C [20].

The crude protein content was obtained from total nitrogen determination by Kjeldahl method based on that most proteins of the studied samples are composed by 16% of nitrogen. The fat content was determined by accelerated solvent extraction (ASE) by solubilization of the ether soluble compounds. These procedures are based on AOAC International [21].

Sample masses of 200 mg were microwave-assisted digested using 2 mL HNO_3 solution in different concentrations (14, 7 and 2 mol L^{-1}) plus 1 mL of H_2O_2 of 30% (w/v) in a closed vessel. After decomposition, digested solutions were transferred to volumetric flasks and diluted with water to 15.0 mL. The microwave oven heating program was performed in five steps: (1) 2 min at 250 W; (2) 2 min at 0 W; (3) 4 min at 650 W; (4) 5 min at 850 W; and (5) 5 min at 1000 W.

Elemental and residual carbon contents were determined by ICP OES with radial view configuration. In order to remove volatile carbon compounds, digests were heated for 20 min at 120 °C [1] and afterwards purged with N_2 to remove dissolved CO_2 and minimize interferences. The instrumental parameters adopted were: 40 MHz generator frequency; 1.3 kW RF power; 15 L min^{-1} plasma gas-flow rate; 1.5 L min^{-1} auxiliary gas-flow rate; 0.9 L min^{-1} nebulizer gas-flow rate; cyclonic nebulization chamber; concentric nebulizer; 0.8 mL min^{-1} sample flow rate; and emission wavelengths (nm):

Table 1
Original contents of carbon, hydrogen, nitrogen, sulfur, crude protein (CP) and ether extract (EE) for the evaluated samples (mean ± standard deviation, $n = 3$).

Sample	N (%)	C (%)	H (%)	S (%)	Crude protein (%)	Ethereal extract (%)
Bovine blood	13.6 ± 0.2	47.2 ± 0.6	7.2 ± 0.8	0.75 ± 0.1	80.5 ± 0.4	1.6 ± 0.29
Bovine muscle	13.2 ± 0.1	50.1 ± 0.8	7.6 ± 0.4	0.81 ± 0.1	80.1 ± 0.43	10.8 ± 0.14
Soybean	7.1 ± 0.3	50.9 ± 0.5	7.8 ± 0.3	0.42 ± 0.1	41.4 ± 0.01	20.4 ± 0.74
Bovine viscera	3.2 ± 0.8	65.6 ± 0.9	10.2 ± 0.7	–	18.9 ± 0.67	63.7 ± 0.31

C(I) 193.025; Ca(II) 396.847; Fe(II) 279.553; K(I) 766.491; Mg(II) 280.275; Na(II) 588.995; P(I) 213.628; and Zn(I) 202.549.

2.3.2. Sample preparation for ^1H NMR analysis

After performing microwave-assisted digestions, the organic remaining compounds were extracted using ethyl ether (2.0 mL). Then, the organic phase was evaporated at 30 °C and the residues were reconstituted in deuterated methanol (0.8 mL) and tetramethylsilane (TMS) for ^1H NMR analysis.

2.3.3. Sample preparation for HPLC analysis

After performing microwave-assisted digestions, the organic remaining compounds were extracted using a liquid–liquid procedure. A volume of 3.0 mL of the digested solution was mixed by vortex action for 1 min with ethyl ether (2.0 mL). Then, the organic phase was rotary evaporated at 30 °C and the residue was reconstituted in 1.0 mL of methanol. Solutions were mixed by vortex action for 15 s and aliquots of 200 μL were transferred to auto-sampler vials and finally 10 μL volumes were injected into the HPLC system.

3. Results and discussion

3.1. Elemental analysis (C, H, N and S), crude protein (CP) and ether extract (EE)

The contents of C, H, N and S, crude protein and ether extract for the original samples, bovine blood and viscera, soybeans grains and the SRM muscle (NIST 8414), are presented in Table 1.

The initial carbon content varied from 47% to 65%, and the greatest concentration was found in bovine viscera sample. Nitrogen amounts were greater in bovine blood and bovine muscle samples, both around 13%. Bovine viscera samples presented the greatest content of ether extract, followed by soybeans grains, which also presented intermediate value of protein, around 42%. Blood and bovine muscle presented the greatest contents of crude protein, around 81%.

3.2. Residual carbon content and element recoveries

The RCC values obtained for each investigated sample are presented in Fig. 1.
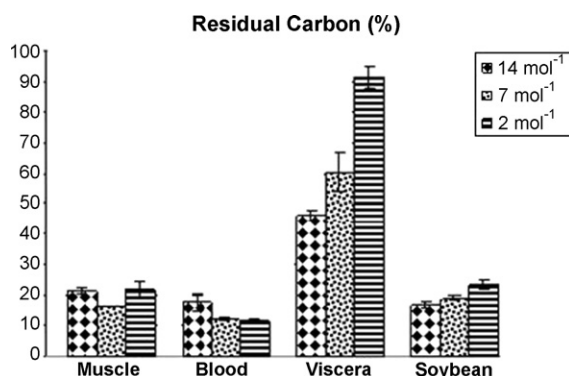

Fig. 1. Residual carbon content (%) in digested samples with different oxidant mixtures.

It can be seen for viscera sample containing around 64% of EE that a decrease in acid concentration led to an increase in RCC values. The use of a solution containing 2 mol L^{-1} HNO$_3$ was not effective for promoting complete digestion of viscera samples, resulting in RCC of 90%. Solid residues and a yellowish color were observed during for viscera digest due to its incomplete decomposition. The same effect was observed for soybeans grains, containing around 21% of EE. However, this effect was less pronounced when compared to bovine viscera. Differences of structural lipids between animal and vegetable samples would be a possible explanation. Samples digested with concentrated nitric acid solutions presented greater standard deviations. On the other hand, for samples containing greater amounts of crude protein an opposite effect was observed. A decrease of acid concentration led to a decrease in RCC from 22% to 15%. The SRM bovine muscle presented RCC of 23% when decomposed using a 14 mol L^{-1} of HNO$_3$ solution. On the other hand, solutions containing 2 mol L^{-1} HNO$_3$ resulted in similar RCC, around 22%

Temperature is the principal factor for evaluating sample digestion procedures. In this study the temperatures reached in all samples were: 160–170 °C for 14 mol L^{-1}; 170–180 °C for 7 mol L^{-1} and 180–200 °C for 2 mol L^{-1} nitric acid concentrations. The hypothesis to explain the decrease in RCC when diluted nitric acid solution was employed is related to the observed increment in temperature values when the concentration of water molecules increased in the solution. However, diluted acid solutions were inefficient for digesting viscera samples containing the greatest amount of fats in their composition.

A certified reference material was employed for evaluating accuracy. Determined and certified concentrations are shown in Table 2. For calcium and magnesium amounts in SRM bovine muscle, it was verified that suitable recoveries occurred when a 2 mol L^{-1} HNO$_3$ solution was employed. It was observed that for Fe and Zn, digestions with diluted acid solutions presented results closer to the certified values. Accuracy was improved when using a 2 mol L^{-1} HNO$_3$ solution for digestions. The use of 7 mol L^{-1} of HNO$_3$ solution presented RCC of 16% and proper mineral recoveries with low RCC and smaller deviations when compared to digestions using concentrated HNO$_3$.

The use of diluted solutions can prevent the formation of insoluble salts, and improve the solubility of some minerals [11].

3.3. Identification of reaction products by ^1H NMR

Considering ^1H NMR spectra obtained for digests after decomposition using 14 and 7 mol L^{-1} HNO$_3$ for blood and bovine muscle samples, the presence of organic residues in the aliphatic region (δ 0.5–3.0 ppm) was confirmed, as well as intense and abundant signal in the typical region of nitro-aromatics compounds (δ 7.0–10.5 ppm; Fig. 2). Different amounts and lower variety of organic compounds were verified when oxidant mixtures were compared. At high concentration, high pressure and high temperature, it was observed an increase in the oxidant power of the reaction mixtures, which oxidizes a greater amount of compounds, generating a wider variety of organic residues.

Digests of bovine viscera obtained using 7 mol L^{-1} of HNO$_3$ were analyzed by ^1H NMR, and it was observed peaks in the δ

Table 2
Mineral concentration in samples digested with 2 mL of HNO_3 in different concentration plus 1 mL of H_2O_2 (30% w/v; mean ± standard deviation, $n = 3$).

		Mineral concentration ($mg\,kg^{-1}$)						
		Ca	Fe	K	Mg	Na	P	Zn
Bovine muscle ($mol\,L^{-1}$)								
Certified		145 ± 20	71.2 ± 9.2	10517 ± 37	960 ± 95	2100 ± 80	8360 ± 450	142 ± 12
Found	14	131 ± 14	50 ± 8	10609 ± 838	1089 ± 34	1491 ± 42	9640 ± 204	174 ± 12
	7	155 ± 10	75 ± 7	11587 ± 570	1113 ± 27	1957 ± 30	8703 ± 192	149 ± 10
	2	143 ± 11	69 ± 6	10145 ± 410	1031 ± 17	2031 ± 17	10655 ± 162	138 ± 11
Bovine blood	14	174 ± 4	1692 ± 57	745 ± 22	56 ± 2	5899 ± 222	860 ± 93	198 ± 3
	7	196 ± 4	1867 ± 19	683 ± 11	64 ± 2	6414 ± 127	896 ± 73	236 ± 8
	2	199 ± 8	1942 ± 14	693 ± 20	66 ± 3	6624 ± 110	907 ± 77	249 ± 4
Soybean	14	1874 ± 44	113 ± 10	10870 ± 389	2629 ± 37	116 ± 24	6207 ± 231	19 ± 4
	7	1960 ± 40	133 ± 15	11286 ± 136	2847 ± 55	78 ± 10	6351 ± 103	46 ± 4
	2	2005 ± 44	129 ± 14	11694 ± 196	2981 ± 43	100 ± 12	6889 ± 254	53 ± 5
Bovine viscera	14	2510 ± 281	99 ± 2	1810 ± 76	134 ± 10	1021 ± 30	3646 ± 185	11 ± 3
	7	2382 ± 762	117 ± 2	1644 ± 72	133 ± 17	1030 ± 34	3638 ± 377	18 ± 2
	2	2437 ± 189	–	1508 ± 97	140 ± 14	868 ± 96	3689 ± 377	21 ± 4

8.4 ppm region, evidencing the presence of nitro-compounds. A signal at δ 5.2 ppm in digests obtained using 2 mol L⁻¹ HNO_3 could be attributed to the presence of water in the sample (δ 4.7 ppm), overlapping with other peaks as, for example, free fatty acid, which present a typical signal at δ 5.3 ppm [22]. It may be inferred that

diluted nitric acid solution did not have sufficient strong oxidative action for bovine viscera sample digestion.

For soybeans grains samples, differences can be observed in the nitro-derivative aromatic compound region (δ 7.0–10.5 ppm; Fig. 2). The signal at δ 5.2 ppm was not observed for this sample. In all
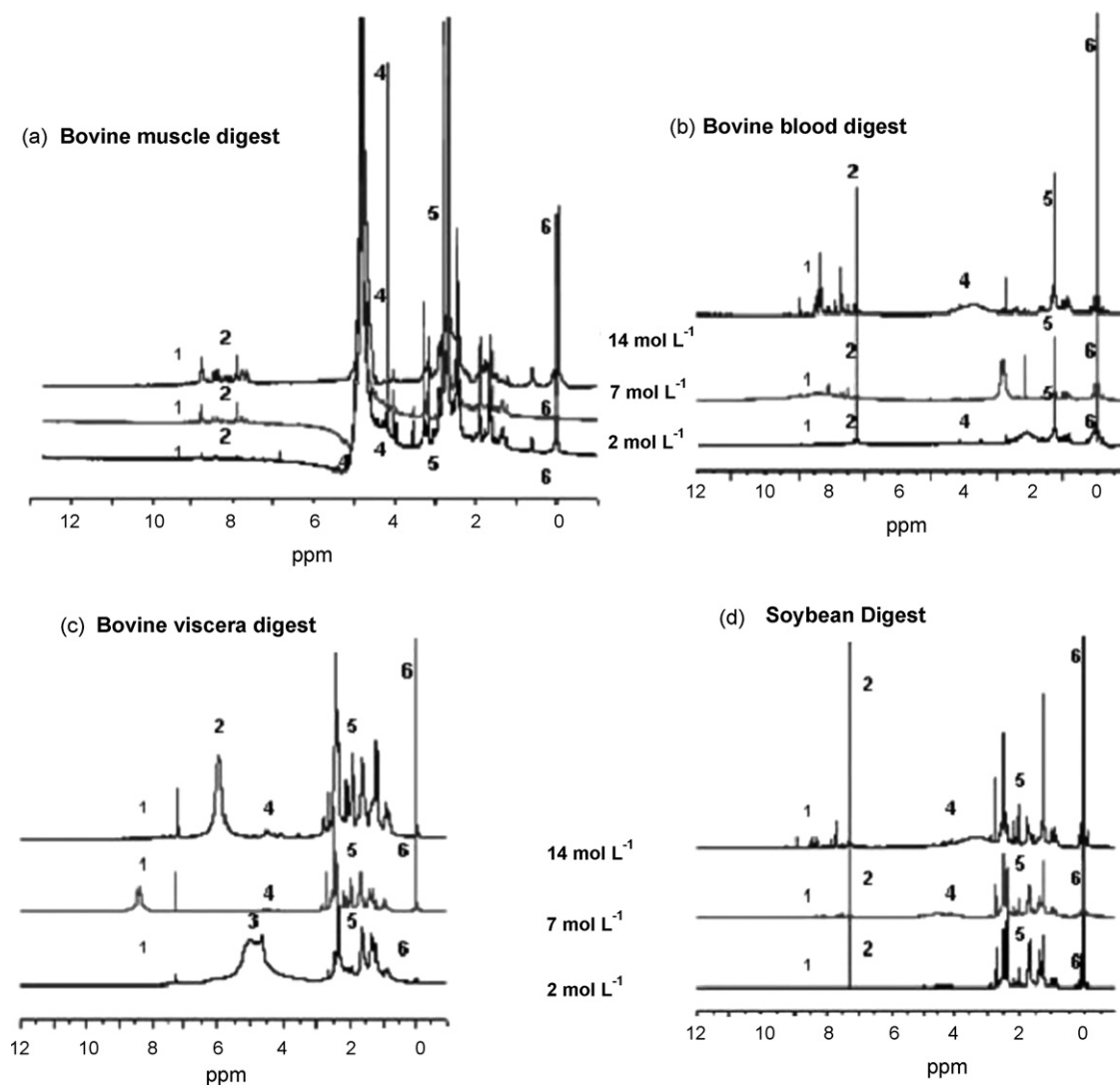
Fig. 2. ¹H NMR spectra of the organic residue of digested samples: (1) aromatic; (2) aliphatic nitro-compounds; (3) hydrogens on doubly bonded from unsaturated fatty acids; (4) carbinolic hydrogens; (5) aliphatic; (6) TSS signal.
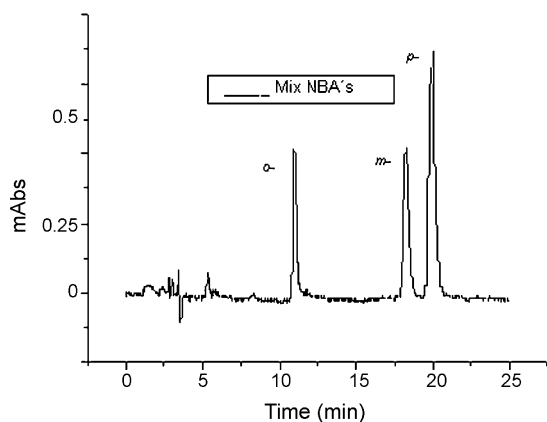
Fig. 3. Chromatograms for standard mixture of *o*-, *m*- and *p*-nitrobenzoic acid. Chromatographic conditions: C_{18} column with water:acetonitrile:methanol (75:20:5, v/v/v) + 0.05% TFA as mobile phase, 1.0 mL min^{-1} of flow-rate, 10 μL of volume injection and 254 nm of detection wavelength.

cases, small peaks, probably carbinolic hydrogen, were observed at δ 4.0–4.7 ppm region [19].

3.4. Separation of reaction products by HPLC-UV

Preliminary experiments were based on the use of different mixtures of water:acetonitrile; water:methanol and water:acetonitrile:methanol as mobile phase for retention of all three nitrobenzoic acid isomers. However, these compounds were not enough retained under any evaluated conditions. In order to elucidate the influence of ion pair additives in the mobile phase and according to a previous work by Chen and Zhang [22], a modified mobile phase employing trifluoracetic acid (TFA) as ion pair was evaluated to simultaneously separate all target isomers of nitrobenzoic acid. Two different proportions of eluent were used to achieve efficient separation of the compounds under isocratic mode, water:acetonitrile:methanol (70:20:10, v/v/v) + 0.05% (v/v) TFA and water:acetonitrile:methanol (75:20:5, v/v/v) + 0.05% (v/v) TFA. This later mobile phase led to effective separation and appropriate retention times for *o*-, *m*- and *p*-nitrobenzoic acids within 25 min analysis time.
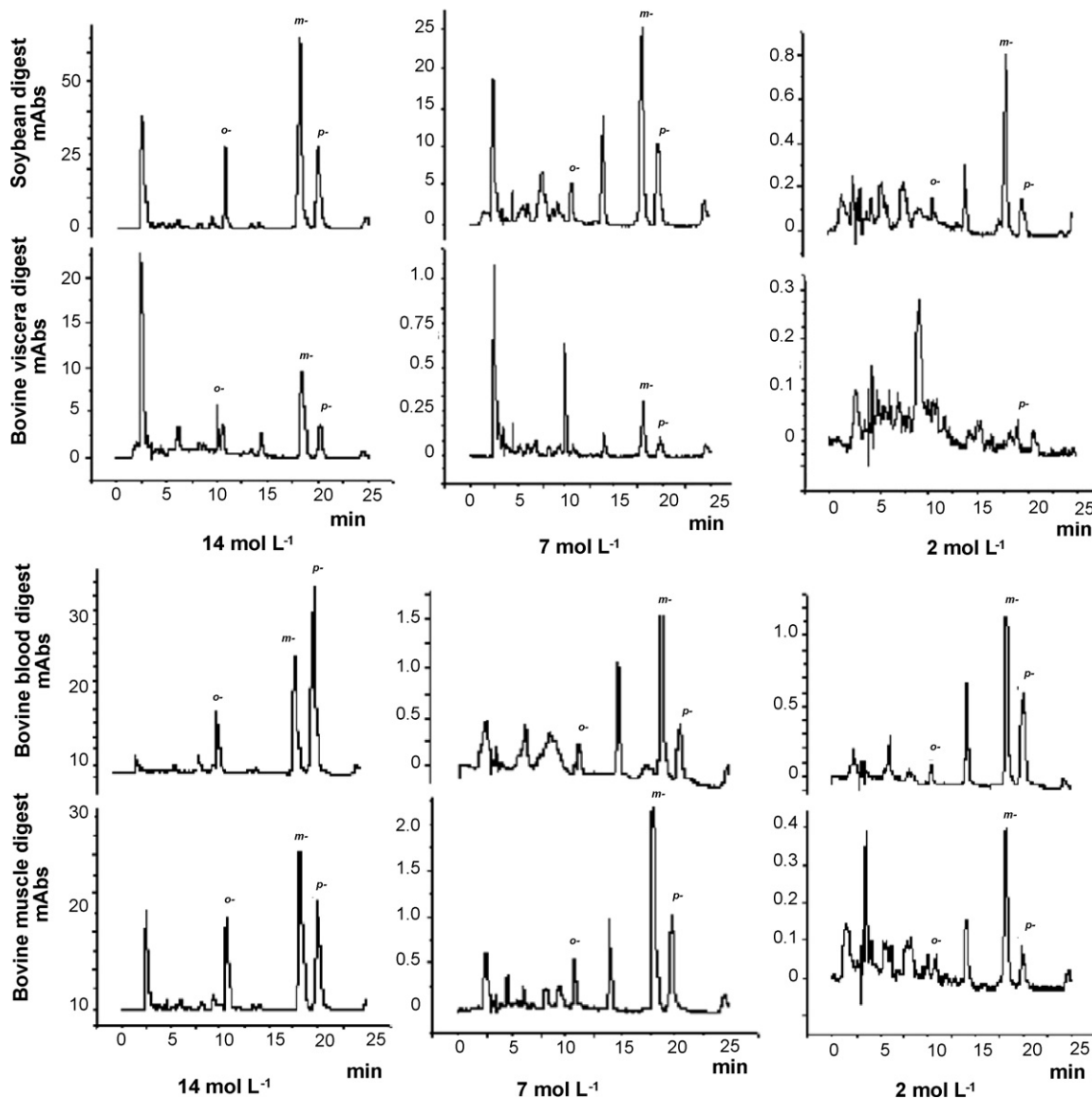

Fig. 4. Chromatograms for bovine muscle, blood, viscera and soybean grains. Chromatographic conditions: C_{18} column, water:acetonitrile:metanol (75:20:5, v/v/v) + 0.05% TFA as mobile phase, 1.0 mL min^{-1} of flow-rate, 10 μL of volume injection and 254 nm of detection wavelength.

The improvement of the separation of the three isomers by adding an ion pair additive into the mobile phase improved the separation of all compounds, and this effect might be explained considering the ionization suppression of the acid group and a possible ion pair formation between the cationic portion of the molecules and the anionic portion of the TFA. The chromatogram obtained for the three isomers at 5 μg mL^{-1} concentration is shown in Fig. 3. The retention times of *o*-NBA, *m*-NBA and *p*-NBA were 10.9, 18.2 and 19.9 min, respectively.

The chromatograms of bovine muscle and blood digested using three different nitric acid concentrations for digestions are shown in Fig. 4. It may be seen that lower contents of NBA isomers were formed when employing diluted acid solutions. The isomers species were identified by matching the retention time of the chromatographic peaks with those of the standards.

Similar characteristics were observed for bovine viscera and soybeans grains (Fig. 4). The *o*-NBA is absent in viscera digested using 2 and 7 mol L^{-1} of nitric acid solutions, and only *p*-NBA appears in the viscera decomposed with 2 mol L^{-1}.

Comparing the digested solutions with the original sample compositions, biological matrices with structural amino acids, proteins and lipids produced nitrobenzoic acid isomers and other organic compounds owing to the cleavage of chemical bonds. The *m*-nitrobenzoic acid was formed in most digestion conditions.

Results obtained in this study are in agreement with the literature. The mechanism of decomposition is similar in almost all cases, generating a mixture of NBA's. These results extend the previous study published by Pratt et al. [14], since these authors concluded that the formation of the three isomers indicates that the dominant mechanism in microwave-assisted digestion is the nitration of the aromatic ring followed by oxidation of the amino acid side chain for bovine liver samples. Daniel et al. have shown that for vegetable samples (NIST SRM peach leaves), the isomers of nitrobenzoic acid are the major products at temperatures around 180 °C [17]. Würfels et al. described that there is a quantitative degradation of saturated and monounsaturated chains of C–H and, in contrast, there is not a complete mineralization of polyunsaturated fat in HNO$_3$ medium [15]. Aliphatic and aromatic acids, nitro-compounds, oxalates and inorganic nitrates and phosphates were found as a decomposition product in different samples analyzed by Reid et al. [16].

Based on green chemistry principles, the use of diluted acids for digestions led to relevant advantages, such as cost reduction, minimization of residues, reduction of blank values, and prevention of damages to components of the equipment [11,19].

4. Conclusions

The use of diluted acids was proven to be a feasible and recommendable alternative, reducing the volume of the reagents and the amount and variety of digestion residues.

The ^{1}H NMR spectra obtained for digests in diluted nitric acid medium are simpler than those obtained when using 14 mol L^{-1} HNO$_3$. Concentrated solutions presented a broader variety of organic compounds, such as nitro-derivative aromatic compounds, and a higher amount of aromatic aliphatic compounds.

The separation of isomers *o*-, *m*- and *p*-nitrobenzoic acid was possible employing ion pair reversed phase HPLC-UV. The qualitative method developed allowed to evaluate different digests. Taking into account the effectiveness of digestions of complex samples with diluted nitric acid solutions one may wonder why this approach has not been used before. Probably this can be explained by the relatively recent availability of closed reaction vessels that can withstand high pressure and high temperature. Additionally, these vessels have enough volume to contain oxygen gas in sufficient amount to promote the regeneration of nitric acid according to the mechanism proposed. Last but not least, these vessels also present an intense temperature gradient in the first step of the heating program which favors condensation of gas products formed by oxidation reactions.

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