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Determination of alkylphenolic residues in fresh fruits and vegetables by extractive steam distillation and gas chromatography–mass spectrometry

Deng-Kai Yang, Wang-Hsien Ding*

Department of Chemistry, National Central University, Chung-Li 32054, Taiwan

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Abstract

This study describes a simple and sensitive method for determining the alkylphenolic compounds, 4-*tert*-octylphenol (4-*t*-OP), 4-nonylphenol isomers (4-NPs), and their monoethoxylates (4-*t*-OP1EO and 4-NP1EOs), in fresh fruits and vegetables. The method involves extracting a sample by a modified Nielson–Kryger steam distillation extraction using *n*-hexane for 1 h. The alkylphenolic compounds were identified and quantitated by gas chromatography–mass spectrometry (GC–MS) in selected ion monitoring (SIM) mode. Various pH values and amounts of NaCl added to the sample solution were evaluated as extraction conditions. The quantitation limit of this method was less than 0.2 ng/g in 10 g (fresh weight) of sample. Recovery of alkylphenolic compounds in spiked samples exceeded 64% while R.S.D. ranged from 1.0 to 9.8%. Alkylphenolic residues were detected in fresh fruits and vegetables at concentrations of 4-NPs and 4-*t*-OP from n.d. to 16 ng/g and from n.d. to 4.8 ng/g (fresh weight), respectively. NP1EO and OP1EO were always below the quantitation limit.

Keywords: Alkylphenolic residues; Fruits; Gas chromatography–mass spectrometry; Effective steam distillation

1. Introduction

Alkylphenol polyethoxylates (APEOs) are a major class of nonionic surfactants used and produced in Taiwan [1]. It is the most commonly used nonionic surfactants in a range of industrial, household and commercial applications. The use of APEO is banned or restricted in many European countries because their degradation products, 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol isomers (4-NPs), have been demonstrated to be able to mimic natural hormones by interacting with the estrogen receptor [2–6]. These chemicals are popularly known as endocrine-disrupting chemicals (EDCs), environmental hormones [7,8] or hormonally active agents (HAAs) [9]. Higher concentrations of NPE-type residues in Taiwanese rivers and sediments than in other countries were detected, because Taiwan's municipal wastewater treatment is deficient [10–13]. Although, the use of APEOs in household detergents has been restricted in many countries, but they are still used as emulsifying agents in latex paints [14] and 4-NPs are used to yield tris(nonylphenol)phosphite (TNPP) as antioxidant stabilizers in plastics [15]. In general. TNPP may be hydrolyzed under acidic conditions to produce 4-NPs and hydrogen phosphite. In this way, some 4-NPs can be generated during the use of TNPP and may migrate into fruits and vegetables. Moreover, surfactants or surfactant mixtures (non-ionic, anionic and cationic surfactants) are added to pesticide formulations to enhance the stability of pesticide suspensions and emulsions [16]. They increase the leaf retention of spray solution [17]; enhance herbicide effectiveness [18,19], and promote the adhesive forces of aqueous droplets on the surface of crop leaves [20]. Following their applications, the degradation products of APEOs (such as 4-NPs and 4-*t*-OP) could be accumulated on the peel of the fruits and vegetables. The presence of endocrine-disrupting residues in the environment or food has raised increasing concern about their impact on wildlife and human health. One possible route of direct human exposure involves the residues of such compounds in the peel of fruits and vegetables. However, data on the concentration of alkylphenolic residues in food are scarce, especially for fresh fruits and vegetables [21]. Therefore, the widespread use of APEOs as emulsifying agents, and increasing public concern over

* Corresponding author. Tel.: +886 3 4227151x65905; fax: +886 3 4227664.
E-mail address: wding@cc.ncu.edu.tw (W.-H. Ding).

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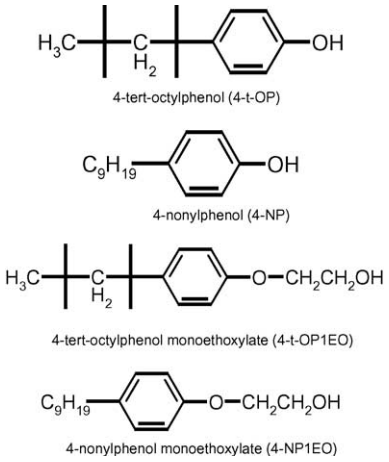

Fig. 1. Structures of alkylphenolic compounds of interest in this study.

public health have motivated this investigation of the content of alkylphenolic residues in fresh fruits and vegetables.

Many analytical methods have been developed for determining alkylphenolic residues in aqueous and solid samples. They have been extensively reviewed by Thiele et al. and Lee (see [22,23] and the references cited therein). The extraction of alkylphenolic compounds from solid samples, such as sewage sludge, sediments, and soils is commonly performed by Soxhlet extraction, extractive steam distillation, supercritical fluid extraction [22,23], and the newly developed pressurized liquid extraction (PLE) technique [12]. The steam distillation method has also been implemented to extract alkylphenolic residues from various foodstuffs [21] and seafood [24–26]. This modified Nielson–Kryger steam distillation method for extracting organics from solid samples, based on the vapor pressure and the solubility of the analytes in the water, has been reported to be a simple and highly efficient method for extracting semi-volatile compounds from solid materials [27]. This method also minimizes the coextraction of interfering molecules and requires far less solvent than is required by Soxhlet extraction.

As part of a wider effort to elucidate the impact of alkylphenolic residues in foodstuffs, this study seeks to develop a reliable means of sensitively detecting and quantitating the concentration of alkylphenolic compounds, 4-*tert*-octylphenol (4-*t*-OP), 4-nonylphenol isomers (4-NPs), and their monoethoxylates (i.e., 4-*t*-OP1EO and 4-NP1EOs) (Fig. 1), in fresh fruits and vegetables. This study incorporates a preliminary investigation of the alkylphenolic residues in food, to help to estimate the daily intake of alkylphenolic compounds.

2. Experimental

2.1. Chemicals and reagents

Unless stated otherwise, all high purity chemicals and solvents were purchased from Aldrich (Milwaukee, WI, USA), Tedia (Fairfield, OH, USA) and Merck (Darmstadt, Germany), and were used without further purification. 4-Nonylphenol (4-NP, tech. grade), 4-*tert*-octylphenol (4-*t*-OP, purity > 97%), 4-nonylphenol monoethoxylate (4-NP1EOs, tech. grade), and 4-*tert*.-octylphenol monoethoxylate (4-*t*-OP1EO, purity > 97%) were purchased from Aldrich. An internal standard [$^2H_{14}$]-*p*-terphenyl (1000 mg/ml) was purchased from ChemServices (West Chester, PA, USA). Stock solutions of each analyte (1000 μg/ml) were prepared in methanol. Mixtures of the analytes for working standard preparation and sample fortification were also prepared in methanol. All stock solutions and mixtures were stored at −10 °C in the dark.

2.2. Sample pretreatment

Seven fresh fruits (apple, nectarine, pear, plum, guava, tomato and grape) and nine fresh vegetables (carrot, cucumber, lettuce, green pepper, broccoli, celery, spinach, mushroom and alfalfa sprout) were purchased from supermarkets in Taiwan. They were analyzed unwashed, with the peel intact, and stoned (i.e., nectarine, pear and apple). The samples were chopped into small pieces, and then homogenized using a food blender (JyeProud JJM9828, Taiwan) just before the extraction. The procedures used for sample extraction of alkylphenolic residues in fruits and vegetables by a small-scale modified Nielson–Kryger steam distillation extraction have been reported elsewhere [24–26], and were used with minor modifications. Briefly, subsample 10 g with 200 ml deionized water was added to a 500-ml round bottom flask with a Teflon^{TM} coated stir bar, then various pH was adjusted between 3.5 and 2.4 by concentrated HC1 with adding various amount of NaCl for "salting out" effect (details are given in Section 3). The flask was then placed onto a heating mantle with a magnetic stir plate below the mantle. Four milliliters of *n*-hexane was added to the column as extraction solvent. The extraction was performed for 1 h and the water layer in the distillation column was discarded. The hexane layer was collected and dried by $MgSO_4$. The extract of alkylphenolic residues was then completely evaporated to dryness using a gentle stream of purified nitrogen. The residues was then redissolved in 100 μl of dichloromethane containing 1.0 ng/ μl of [$^2H_{14}$]-*p*-terphenyl (as an internal standard), and made ready for GC–MS analysis.

The recovery experiments were performed using the spiked samples. Concentration of 1.0 μg/ml of each 4-*t*-OP and 4-*t*-OP1EO, and total concentration of 1.0 μg/ml of 4-NPs and 4-NP1EOs standard mixture in 100 μl of methanol were carefully distributed on top of the blended samples by a glass syringe. The samples were mixed by tumbling for 30 min. The spiked samples (final concentration 10 ng/g) were then stored in a tightly closed brown glass vials at room temperature for 24 h, and made ready for spiked experiments. For recovery experiment, spiked samples were followed the extraction and GC–MS measurement procedures, and then the recovery was calculated by comparing the con-

centrations obtained and the spiked known amount of the analytes.

2.3. GC–MS analysis

Analyses were performed on a HP-5890 Series II gas chromatograph directly coupled to a HP-5973 mass selective detector (Hewlett-Packard, Delaware, USA) operating in electron impact (EI) and selected ion monitoring (SIM) modes. Samples (1 μl) were injected with the injection temperature at 300 °C in the splitless mode. A DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film, J&W, CA, USA) was used. The GC temperature program was as follows: 70 °C for 2 min, followed by a temperature ramp at 30 °C/min to 130 °C, then temperature ramp at 8.5 °C/min to 300 °C, and hold for 5 min. The transfer line was set at 280 °C. Quantitation of the analytes was carried out in the selected ion monitoring (SIM) mode. The selected masses were *m*/*z* 107, 135 and 149 for 4-NPs; *m*/*z* 135 and 107 for 4-*t*-OP; *m*/*z* 135 and 179 for 4-*t*-OP1EO; as well as *m*/*z* 135, 179 and 193 for 4-NP1EOs at dwell time of 100 ms/ion/scan. The detector was tuned with perfluorotributylamine (PFTBA) by using the autotune program. The electron energy was 70 eV, and the electron multiplier was operating at 200–300 V above the autotune value with the high energy dynode on.

3. Results and discussion

According to our previous report [26], compared to Soxhlet extraction and pressurized liquid extraction (PLE) techniques, extractive steam distillation is a more simple and effective method for extracting alkylphenol residues from biological tissue samples. In this work, various extraction conditions (i.e., pH and the amounts of NaCl) of extractive steam distillation were investigated in more detailed for extracting alkylphenolic residues from fruit and vegetable samples. Fig. 2 reveals that by adjusting pH of a spiked sample solution of apple to 3.5 (condition A) or 2.7 (condition B) and adding 0.1 g NaCl, maximum recoveries (67–87%) could be achieved. When pH was reduced to 2.4 (condition C), the recovery of 4-*t*-OP1EO (57 ± 6%) was significantly reduced. Furthermore, the effect of NaCl was also investigated at pH 2.7, by adding 0.2 g NaCl to the sample solution; lower recoveries of 4-*t*-OP1EO (51 ± 3%) and 4-NP1EOs (53 ± 4%) were observed (condition D). However, when NaCl was not added to the sample solution, the recoveries of all analytes (33–66%) were markedly lower (condition E). Moreover, when the sample solution was not acidified to reduce the pH (original pH = 4.4), and when 0.1 g NaCl (condition F) or no NaCl was added (condition G), lower recoveries (41–85%) were obtained. These results indicate that the best conditions for extracting of alkylphenolic residues from fruits and vegetables were achieved by acidified sample solution to pH 2.7 and adding 0.1 g of NaCl for "salting out" effect.

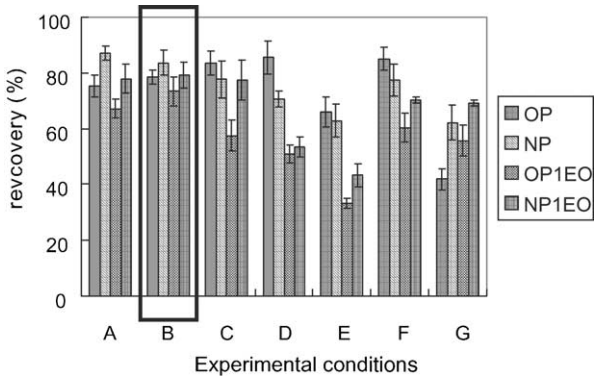

Fig. 2. Extraction recoveries of alkylphenolic compounds from a spiked apple sample in various conditions, the extraction conditions were described in text. Extractions were performed on three replicates, standard deviation is reported as an error bar.

All residues in concentrations are reported on a fresh weight basis. The quantitation limit of this method was less than 0.2 ng/g in 10 g (fresh weight) of the sample. Quantitation for 4-NPs and 4-NP1EOs was based on the sum of the peak areas for all isomers, and was calculated from the five-point calibration curve, as indicated by the response factors, covering the range 0.1–10 μg/ml, each divided by the fixed concentration of an internal standard [28,29]. The precision of the calibration curve, as indicated by the relative standard deviation (R.S.D.) of response factors, was 7% and 10%, 9% and 10%, for 4-*t*-OP, 4-NPs, 4-*t*-OP1EO and 4-NP1EOs, respectively. The correlation coefficients exceeded 0.998. The curves covered a range equivalent to the concentration of the analytes in the final extract.

The method validation was carried out via recovery study by using three replicate 10 g subsamples spiked to yield the final concentrations of 10 ng/g for each of 4-*t*-OP and 4-*t*-OP1EO, and 10 ng/g for each total 4-NPs and 4-NP1EOs. The precision of the method, as indicated by the relative standard deviation (R.S.D.) of the recovery, was assessed using three independent pretreatment and extractions of analytes from samples. Average recovery ranged from 64 to 103% with R.S.D. ranging from 1 to 10%, indicating good recovery and repeatability of the method (Table 1). Fig. 3 displays typical SIM chromatograms of 4-*t*-OP and 4-NP isomers detected in the samples of (a) a pear, and (b) a broccoli. The peaks were identified and quantitated using retention times with their characteristic ions of SIM and response factors, respectively. Positively identified target compounds in every fruit and vegetable were analyzed three replicate. Table 2 indicates that 4-NP isomers were detected in all the fruit samples, except guava, at concentrations from 3.7 to 16 ng/g (fresh weight). No alkylphenolic residues were detected in most selected vegetables, except broccoli, and the concentrations of 4-*t*-OP and 4-NPs were 0.4 and 4.8 ng/g (fresh weight), respectively. 4-*t*-OP1EO and 4-NP1EOs were always below the quantitation limit. The varying concentrations of 4-*t*-OP and 4-NPs reveal that these compounds had found their way into food through miscellaneous pathways and at different stages

Table 1
Recovery of alkylphenolic compounds in spiked fruits and vegetables

Sample	Recovery (%)			
	4-*t*-OP	4-NPs	4-*t*-OP1EO	4-NP1EOs
Fruits				
Apple	89[a] (1.5)[b]	98 (1.0)	92 (9.6)	83 (1.4)
Nectarine	64 (4.7)	92 (3.5)	100 (2.5)	100 (9.0)
Pear	78 (1.8)	87 (5.7)	92 (2.1)	84 (2.1)
Grape	91 (4.6)	84 (1.7)	96 (3.2)	83 (6.5)
Plum	72 (9.6)	77 (4.7)	94 (6.9)	84 (6.0)
Guava	84 (1.8)	81 (7.5)	92 (2.1)	82 (2.1)
Tomato	101 (2.9)	91 (1.6)	97 (9.7)	96 (9.8)
Vegetables				
Carrot	101 (5.3)	85 (3.0)	101 (3.4)	75 (3.4)
Cucumber	93[c]	88	99	75
Lettuce	69	97	97	99
Green pepper	100	106	72	86
Broccoli	96 (4.8)	81 (1.1)	92 (2.6)	79 (1.5)
Celery	77	89	100	71
Spinach	65	92	92	100
Mushroom	101 (2.0)	87 (1.0)	97 (2.6)	89 (1.5)
Alfalfa sprout	87	73	98	88

[a] The average spiked recovery ($n = 3$).
[b] The relative standard deviations (R.S.D.%) of spiked recovery are given in parentheses.
[c] The spiked recovery ($n = 1$).

Table 2
Concentration of alkylphenolic compounds detected in fruits and vegetables

Sample	Concentration (ng/g)			
	4-*t*-OP	4-NPs	4-*t*-OP1EO	4-NP1EOs
Fruits				
Apple	n.d.	5.9[a] (4.5)[b]	n.d.	n.d.
Nectarine	n.d.	3.9 (7.2)	n.d.	n.d.
Pear	0.7[a] (1.9)[b]	7.6 (2.0)	n.d.	n.d.
Grape	n.d.	3.7 (9.1)	n.d.	n.d.
Plum	n.d.	16 (8.5)	n.d.	n.d.
Guava	n.d.	n.d.	n.d.	n.d.
Tomato	n.d.	5.9 (4.5)	n.d.	n.d.
Vegetables				
Carrot	n.d.	n.d.	n.d.	n.d.
Cucumber	n.d.	n.d.	n.d.	n.d.
Lettuce	n.d.	n.d.	n.d.	n.d.
Green pepper	n.d.	n.d.	n.d.	n.d.
Broccoli	0.4 (7.6)	4.8 (5.2)	n.d.	n.d.
Celery	n.d.	n.d.	n.d.	n.d.
Spinach	n.d.	n.d.	n.d.	n.d.
Mushroom	n.d.	n.d.	n.d.	n.d.
Alfalfa sprout	n.d.	n.d.	n.d.	n.d.

[a] The average concentration (ng/g) detected in the samples ($n = 3$).
[b] The relative standard deviations (R.S.D.%) of detected concentration are given in parentheses. n.d.: not detected at limits of quantitation (LOQ).

of the food production process. Some may originate from APEOs, which are used as nonionic surfactants in disinfectants or as emulsifiers in pesticide formulations. Following their use in agriculture, the degradation products of APEOs could promote the accumulation of 4-*t*-OP and 4-NPs on the peel of the fruits and vegetables. Another possible source might be from the plastic food-contact materials, which contain tris(nonylphenol)phosphite (TNPP) as antioxidant stabi-

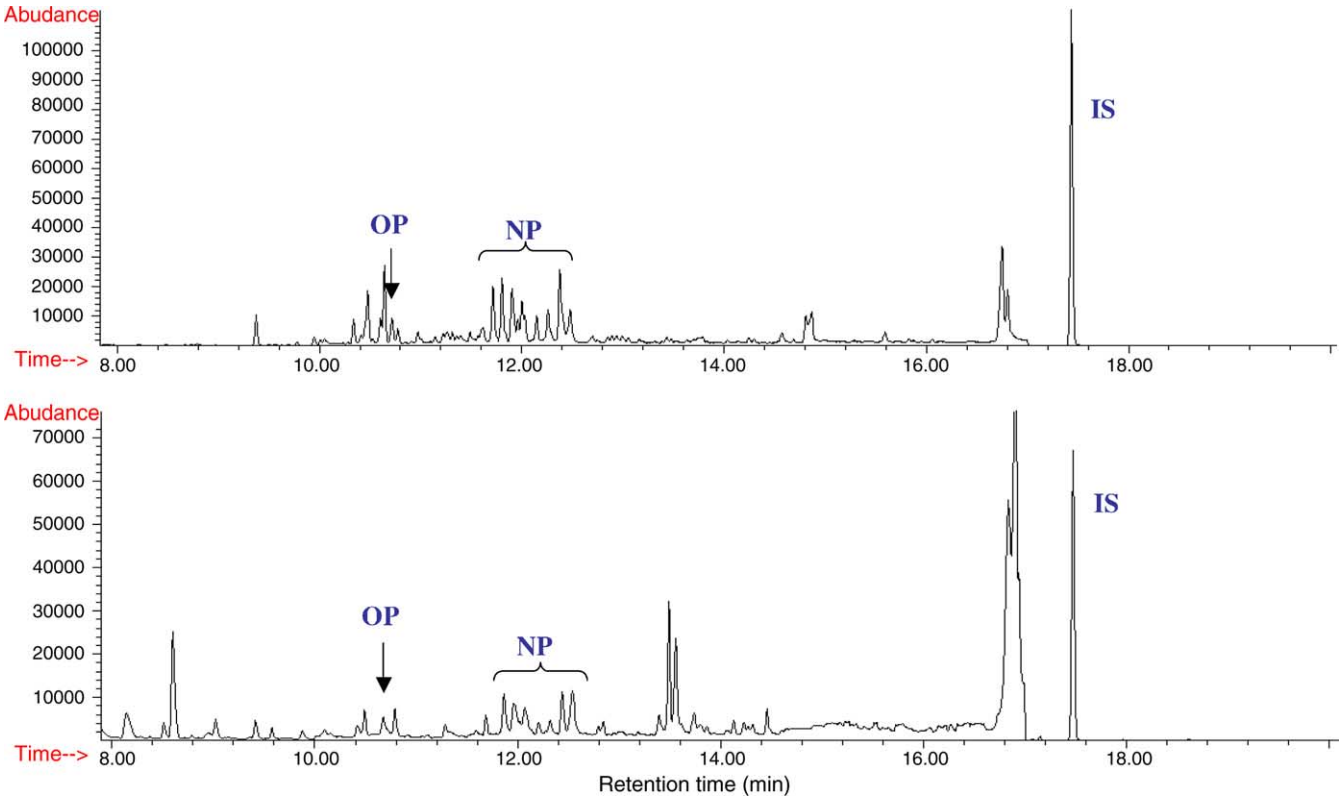

Fig. 3. Typical SIM chromatograms of 4-*t*-OP and 4-NP isomers from (a) a pear sample and (b) a broccoli sample.

lizers. TNPP is manufactured by the reaction of 4-NPs with phosphorous trichloride. In this way, the degradation products or the impurities of 4-NP residues from TNPP in the plastic may migrate into fruits and vegetables when used in food-contact applications [15].

4. Conclusion

The analytical procedures developed herein reveal that extractive steam distillation and GC–MS are reliable and sensitive methods that are convenient analytical techniques for detect traces of alkylphenolic residues in fruits and vegetables. Extractive steam distillation minimizes the co-extraction of other compounds from fruits and vegetables while providing detection limits that suffice to determine toxicologically relevant concentrations. Preliminary results show that 4-NP residues are ubiquitous in fruits sold in Taiwan. Consequently, the content of these residues must be routinely monitored in fruits and vegetables, and reported to satisfy consumer health and food safety concerns.

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