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# Comparative Study on Extraction Methods of Free and Glycosidically Bound Volatile Compounds from Kaffir Lime Leaves by Solvent Extraction and Solid Phase Extraction

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## ABTRACT

Two extraction methods, solvent extraction and solid phase extraction using Amberlite XAD-2 resin, were used to extract free and glycosidically bound volatile compounds from fresh kaffir lime leaves. Better result was obtained with the solvent extraction method. Fifty-four free and thirty-nine glycosidically bound volatile compounds were obtained by solvent extraction method and their odor descriptions related to kaffir lime leaf odors. With the XAD-2 resin, fifty free and ten glycosidically bound volatile compounds were obtained but most of them did not relate to the volatile compounds in kaffir lime leaves. Most of the free and glycosidically bound volatile compounds extracted with XAD-2 resin had lower concentration than those extracted with solvent extraction, especially the compounds that gave kaffir lime leaf characteristic odor such as citronellal, $\beta$-citronellol, *trans*-geraniol and *trans*-$\beta$-caryophyllene.


**Key words:** solvent extraction, XAD-2 resin, free and glycosidically bound volatile compound, kaffir lime leaves

## INTRODUCTON

Kaffir lime (*Citrus hystrix* D.C., Rutaceae) is a Southeast Asian citrus plant with very strong fragrance. Free volatile compounds in kaffir lime leaf oil distilled by steam distillation have been reported (Lawrence *et al*., 1971). Citronellal is the main volatile compound found in the essential oil of kaffir lime leaves. The other volatile compounds found in kaffir lime leaf oil were $\alpha$-pinene, camphene, $\beta$-pinene, sabinene, myrcene, limonene, *trans*-ocimene, $\gamma$-terpinene, $\rho$-cymene, terpinolene, copaene, linalool, $\beta$-cubebene, isopulegol, caryophyllene, citronellyl acetate, citronellol, geranyl acetate, $\delta$-cadinene (Lawrence *et al*., 1971). Glycosidically bound volatile compounds of fresh kaffir lime leaves have not been studied to date, even if they are the potential precursors of the volatile compounds in many plants. The glycosidically bound volatile compounds are composed of two parts, aglycone and glycone. The anomeric hydroxyl group of the sugar (glycone) is linked to the volatile compound (aglycone) via glycosidic linkage (Chyau *et al*., 2003).

Solvent extraction is one of the simplest approaches for aroma isolation. This method gives a broad range for isolation of volatile compounds, but it consumes large volumes of solvent. Solvent extraction is the most useful method for non-lipid

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food. In the case of food containing lipid, the extract will be re-extracted under high vacuum to separate lipid phase (Reineccius, 2006).

Solid phase extraction is a convenient and popular method. Solid phase, like Amberlite XAD-2 resin, is often used for volatile compounds isolation. XAD-2 resin is a hydrophobic crosslinked polystyrene copolymer resin. Free and glycosidically bound volatile compounds released from XAD-2 resin are fractionated by order of polarity of solvents.

The aim of this study was to compare two isolation methods, (1) solvent extraction and (2) solid phase extraction using XAD-2 resin, for free and glycosidically bound volatile compounds of fresh kaffir lime leaves.

## MATERIALS AND METHODS

### Materials

Kaffir lime leaves were purchased from a market in Bangkok. They were cleaned with distilled water, wiped to dry, and trimmed by cutting out the midrib of the leaves with a scissor.

### Reagents

HPLC-grade solvents and phosphate buffer were purchased from Lab-Scan (Ireland). Sodium chloride and anhydrous sodium sulfate were purchased from Fisher Chemical (England). Internal standards (*tert*-butyl benzene and 2-undecanol), n-alkanes (hydrocarbons, $C_4$-$C_{22}$) and Amberlite XAD-2 resin were purchased from Sigma Aldrich (USA).

### Isolation/extraction

#### Solvent extraction

Kaffir lime leaves (20 g) was blended with 200 mL of liquid nitrogen and NaCl (20 g) in a Waring blender for 20 s. The blended leaves were extracted two times with 80 mL of dichloromethane and two times with 80 mL of pentane, respectively, to isolate free volatile compound. Each extraction was carried out for 30 min. The free volatile compound extract was added with 50 µL of internal standard (*tert*-butyl benzene and 2-undecanol). The mixture was concentrated to 50 mL under the steam of $N_2$ before distillation under $10^{-5}$ torr at room temperature for 2 h. Then, the extract was dried over anhydrous $Na_2SO_4$ (2 g) and concentrated with $N_2$ (gas) to 1 mL. For glycosidically bound fraction, the blended leaves were re-extracted three times with 80 mL of methanol.

**Solid phase extraction: Amberlite XAD-2 resin** (modified from Chyau *et al*., 2003)

Kaffir lime leaves (20 g) was blended with 200 mL of liquid nitrogen in a Waring blender for 20 s. The blended leaves were homogenized with saturated NaCl solution for 5 min and then centrifuged at 6,430 × g, 4°C for 30 min. Methanol 100 mL and deionized (DI) water 100 mL were loaded through XAD-2 column for preconditioning. The clear sample solution was loaded through XAD-2 column at a flow rate of 2.2 mL/min. DI water was loaded to eliminate sugars, acids and other water-dissolved compounds. A 100 mL mixture of pentane/dichloromethane (1:1) was loaded through the column to elute free volatile compounds. After that, a methanol (100 mL) was loaded through the column to elute glycosidically bound volatile compounds. The free volatile compound extract was then added with 50 µL of internal standard and concentrated to 50 mL under the gentle steam of $N_2$ before high vacuum distillation as mentioned above.

**Acid hydrolysis** (modified from Cai *et al*., 2002)

The methanol extracts were concentrated and dried using a rotary evaporator at 50°C and 252 torr. Subsequently, the fractions of glycosidically bound volatile compounds were re-dissolved in a mixture of DI water (100 mL) and phosphate buffer (pH 3.5) 100 mL. The mixtures were hydrolyzed at 50°C for 2.5 h. The aglycones

released from acid hydrolysis were extracted three times with 50 mL of pentane/dichloromethane (1:1) to remove aglycones (30 min/extraction) and a 50 $\mu$L of internal standard was added before high vacuum distillation as mentioned before.

**GC-MS and GC-FID analysis**

The concentrated extract of 1 μL was analyzed on a Hewlett-Packard model HP 6890 GC equipped with a mass selective detector model HP 5973. Capillary column, HP-5 (60 m length, 0.25 mm ID and 0.25 μm film thickness) was used and the carrier gas was helium with a flow rate of 2.2 mL/min. The injector was operated in a split mode (50:1). The oven temperature was initiated at 35°C for 1 min, raised at a rate of 10°C/min to 200°C, and held at this temperature for 10 min. The identity of volatile compounds and their relative index (RI) were done by comparing mass the spectrum with the Wiley 275 library, NIST 98 library and n-alkanes ($C_4$-$C_{22}$) retention index.

The free and aglycone-concentrated extracts were analyzed on a Hewlett-Packard model HP 6890N GC equipped with FID. Capillary column, HP-5 (30 m length, 0.25 mm ID and 0.25 $\mu$m film thickness) was used and the same conditions as using in GC-MS. The relative concentration of compounds was calculated by comparing peak areas with internal standards (*tert*-butyl benzene for monoterpene and 2-undecanol for *l*-linalool).

## RESULTS AND DISCUSSION

**Free volatile compounds**

Solvent extraction is a method in which a sample directly contacted with a solvent. A broad range of volatile compounds was isolated as shown in Table 1. Fifty-four free volatile compounds were identified as aldehydes (78.8%), hydrocarbons (12.2%), alcohols (6.4%), and esters (2.7%). Citronellal (74.8%) was the most abundant compound as reported in the kaffir lime leaf oil extracted by steam distillation, and some compounds such as $\alpha$-pinene, $\beta$-pinene, sabinene, myrcene, limonene, linalool, caryophyllene and citronellol also have been reported (Lawrence *et al.*, 1971). Solvent extracted free volatile compounds with kaffir lime leaf odor such as *l*-limonene, *l*-linalool and $\beta$-citronellol have orange and citrus note.

For solid phase extraction using XAD-2 resin, the identity of free volatile compounds is shown in Table 2. Fifty free volatile compounds were identified as hydrocarbons (90.4%), aldehydes (8.2%), alcohols (1.3%) and ester (0.1%). The abundant compounds were 1-methyl-2-phenylcyclopropane (48.6%) and 4-ethenyl-1,2-dimethylbenzene (22.4%). Free volatile compounds extracted by XAD-2 resin did not show the odor characteristic of kaffir lime leaves and most of them were benzylic compounds that showed the affinity of XAD-2 resin to benzylic compounds as found in the grape isolating by Amberlite XAD-2 (Ibarz *et al.*, 2006). Some compounds that had been reported in kaffir lime leaf oil were not found in XAD-2 method such as $\alpha$-pinene, $\beta$-pinene, sabinene, myrcene, *l*-limonene, *l*-linalool, and citronellyl acetate.

Free volatile compounds found in both solvent extraction and XAD-2 resin methods were shown in Figure 1. Most concentrations of XAD-2 extracted compounds were lower than those of solvent extracts, except for 1-penten-3-ol, *trans*-2-hexanal, and geranial. The concentrations of free volatile compounds that gave the kaffir lime leaf characteristic odor such as citronellal, $\beta$-citronellol, *trans*-geraniol, and *trans*-$\beta$-caryophyllene, extracted by XAD-2 resin were lower than those obtained by solvent extraction.

**Glycosidically bound volatile compounds**

For solvent extraction method, thirty-nine compounds were identified as alcohols (45.3%), aldehydes (33.9%), hydrocarbons (10.5%), and esters (7.8%) (Table 1). Citronellal

**Table 1** Relative concentrations of free and glycosidically bound volatile compounds from fresh kaffir lime leaves isolated by solvent extraction method.

| No. | Compound | RI | Free | | Glycosidically bound | |
|---|---|---|---|---|---|---|
| | | (HP-5) | ppm | % | ppm | % |
| 1 | 1-penten-3-ol | 782 | 2.6 | 0.0 | - | - |
| 2 | 1-penten-3-one | 788 | 3.7 | 0.1 | - | - |
| 3 | 4-pentenal | 845 | 1.7 | 0.0 | - | - |
| 4 | cis-2-pentenol | 869 | 4.5 | 0.1 | 0.2 | 0.1 |
| 5 | hexanal | 900 | 172.1 | 3.2 | 0.5 | 0.2 |
| 6 | *trans*-2-hexenal | 955 | 25.1 | 0.5 | 0.6 | 0.3 |
| 7 | *α*-thujene | 1034 | 0.6 | 0.0 | - | - |
| 8 | *α*-pinene | 1043 | 5.3 | 0.1 | - | - |
| 9 | sabinene | 1082 | 114.0 | 2.1 | trace | trace |
| 10 | *β*-pinene | 1088 | 6.4 | 0.1 | trace | trace |
| 11 | *β*-myrcene | 1094 | 49.2 | 0.9 | - | - |
| 12 | octanol | 1106 | 1.1 | 0.0 | trace | trace |
| 13 | *α*-phellandrene | 1113 | 0.7 | 0.0 | - | - |
| 14 | *δ*-3-carene | 1120 | 1.6 | 0.0 | trace | trace |
| 15 | *l*-limonene | 1138 | 9.7 | 0.2 | - | - |
| 16 | *cis*-*β*-ocimene | 1144 | 1.1 | 0.0 | - | - |
| 17 | *trans*-*β*-ocimene | 1152 | 30.5 | 0.6 | 1.6 | 0.7 |
| 18 | *γ*-terpinene | 1161 | 10.0 | 0.2 | 0.2 | 0.1 |
| 19 | *trans*-sabinene hydrate | 1178 | 7.0 | 0.1 | 0.9 | 0.4 |
| 20 | *α*-terpinolene | 1190 | 2.3 | 0.0 | 0.4 | 0.2 |
| 21 | *l*-linalool | 1204 | 197.1 | 3.6 | 35.4 | 15.6 |
| 22 | *trans*-4,8-dimethyl-1,3,7-nonatriene | 1220 | 3.4 | 0.1 | 0.1 | 0.1 |
| 23 | citronellal | 1264 | 4047.4 | 74.8 | 75.6 | 33.2 |
| 24 | (-)-isopulegol | 1275 | 3.0 | 0.1 | 6.0 | 2.6 |
| 25 | neoiso(iso)pulegol | 1279 | 2.5 | 0.0 | 0.7 | 0.3 |
| 26 | *α*-terpinen-4-ol | 1291 | - | - | 0.7 | 0.3 |
| 27 | *α*-terpineol | 1304 | 0.9 | 0.0 | 0.4 | 0.2 |
| 28 | decanal | 1310 | 1.0 | 0.0 | trace | trace |
| 29 | *β*-citronellol | 1332 | 108.8 | 2.0 | 18.2 | 8.0 |
| 30 | neral | 1351 | 0.6 | 0.0 | 0.1 | 0.0 |
| 31 | *trans*-geraniol | 1359 | 14.2 | 0.3 | 1.1 | 0.5 |
| 32 | geranial | 1378 | 3.1 | 0.1 | 0.2 | 0.1 |
| 33 | 1H-Indole | 1409 | 6.6 | 0.1 | 0.3 | 0.1 |
| 34 | 2-(2-hydroxyl-2propyl)-5-*m*-cyclohexanol | 1452 | - | - | 44.1 | 19.4 |
| 35 | citronellyl acetate | 1455 | 103.2 | 1.9 | 11.4 | 5.0 |
| 36 | geranyl acetate | 1467 | 5.4 | 0.1 | - | - |
| 37 | neryl acetate | 1486 | 28.9 | 0.5 | 5.2 | 2.3 |
| 38 | (+)-cycloisosativene | 1492 | 1.8 | 0.0 | - | - |
| 39 | *α*-copaene | 1497 | 38.5 | 0.7 | 0.9 | 0.4 |
| 40 | *β*-cubebene | 1510 | 32.3 | 0.6 | 0.8 | 0.3 |
| 41 | *trans*-*β*-caryophyllene | 1548 | 181.8 | 3.4 | 7.2 | 3.2 |
| 42 | epi-bicyclosesquiphellandrene | 1555 | 3.1 | 0.1 | 0.1 | 0.1 |
| 43 | *α*-guaiene | 1560 | 1.5 | 0.0 | - | - |
| 44 | *trans*-*β*-farnerene | 1563 | 1.5 | 0.0 | - | - |
| 45 | *α*-humulene | 1583 | 21.7 | 0.4 | 1.3 | 0.6 |
| 46 | (+)-aromadendrene | 1591 | 0.7 | 0.0 | - | - |
| 47 | *γ*-cadinene | 1600 | 4.5 | 0.1 | 0.2 | 0.1 |
| 48 | germacrene D | 1608 | 19.3 | 0.4 | 1.3 | 0.6 |
| 49 | *trans,trans*-*α*-farnesene | 1614 | 28.6 | 0.5 | 2.3 | 1.0 |
| 50 | bicyclogermacrene | 1624 | 52.6 | 1.0 | 3.8 | 1.7 |
| 51 | *δ*-guaiene | 1629 | 0.7 | 0.0 | - | - |
| 52 | *δ*-cadinene | 1644 | 32.6 | 0.6 | 3.7 | 1.6 |
| 53 | nerolidol | 1672 | 11.7 | 0.2 | 1.6 | 0.7 |
| 54 | diethyl phtalate | 1717 | 1.2 | 0.0 | 0.1 | 0.1 |
| | **Total** | | **5,409.3** | | **227.4** | |

**Table 2** Relative concentrations of free and glycosidically bound volatile compounds from fresh kaffir lime leaves isolated by Amberlite XAD-2 resin.

| No. | Compound | RI (HP-5) | Free | | Glycosidically bound | |
|---|---|---|---|---|---|---|
| | | | ppm | % | ppm | % |
| 1 | 1-penten-3-ol | 782 | 3.5 | 0.1 | - | - |
| 2 | 1-penten-3-one | 789 | 4.2 | 0.1 | - | - |
| 3 | 3-methy-2-pentanone | 840 | 4.9 | 0.1 | - | - |
| 4 | 4-methy-2-pentanone | 857 | 13.9 | 0.2 | - | - |
| 5 | toluene | 870 | 8.1 | 0.1 | - | - |
| 6 | hexanal | 900 | 49.1 | 0.8 | 0.4 | 1.6 |
| 7 | 7-oxybicycloheptane | 953 | 3.5 | 0.1 | - | - |
| 8 | *trans*-2-hexenal | 955 | 225.8 | 3.7 | 0.6 | 2.3 |
| 9 | 2,6-dimethyl-2-heptane | 971 | 2.8 | 0.0 | - | - |
| 10 | $\rho$-xylene | 981 | 4.6 | 0.1 | - | - |
| 11 | styrene | 1004 | 348.4 | 5.7 | - | - |
| 12 | 1-ethyl-3-methylcyclohexane | 1014 | 1.9 | 0.0 | - | - |
| 13 | cumene | 1032 | 1.5 | 0.0 | - | - |
| 14 | 1-hexadecanol | 1050 | 0.9 | 0.0 | - | - |
| 15 | n-propylbenzene | 1061 | 6.7 | 0.1 | - | - |
| 16 | 1,2,4-trimethylbenzene | 1070 | 5.0 | 0.1 | - | - |
| 17 | 2,2,4,4-tetramethyl-3-pentanone | 1073 | 7.5 | 0.1 | - | - |
| 18 | 1,2,3-trimethylbenzene | 1076 | 7.5 | 0.1 | - | - |
| 19 | 1-ethyl-3-methylbenzene | 1089 | 1.8 | 0.0 | - | - |
| 20 | 1,3,5-trimethylbenzene | 1131 | 0.8 | 0.0 | - | - |
| 21 | 2-hexyl-1-octanol | 1141 | 4.8 | 0.1 | - | - |
| 22 | styrene oxide | 1154 | 2.9 | 0.0 | 4.0 | 15.6 |
| 23 | 1,3-diethylbenzene | 1159 | 25.9 | 0.4 | 0.7 | 2.7 |
| 24 | 1,4-diethylbenzene | 1165 | 38.3 | 0.6 | - | - |
| 25 | 1,2-diethylbenzene | 1172 | 21.3 | 0.4 | - | - |
| 26 | *trans*-sabinene hydrate | 1178 | 3.5 | 0.1 | - | - |
| 27 | 2-methyl-1-propenylbenzene | 1195 | 26.3 | 0.4 | - | - |
| 28 | 1-methyl-2-phenylcyclopropane | 1197 | 2954.7 | 48.6 | - | - |
| 29 | 4-ethenyl-1,2-dimethylbenzene | 1205 | 1358.6 | 22.4 | - | - |
| 30 | 1-ethyl-4-(1-methylethyl)benzene | 1213 | 13.8 | 0.2 | - | - |
| 31 | divinylbenzene | 1225 | 121.5 | 2.0 | - | - |
| 32 | 1,3-diethyladamantane | 1235 | 85.7 | 1.4 | - | - |
| 33 | 2-ethyl-2,3-dihydro 1H-indrene | 1241 | 15.8 | 0.3 | - | - |
| 34 | 1-methyl-4-(1-methylpropyl)benzene | 1247 | 19.3 | 0.3 | - | - |
| 35 | 1,3-diethyl-5-methylbenzene | 1252 | 13.9 | 0.2 | - | - |
| 36 | citronellal | 1264 | 205.5 | 3.4 | 0.5 | 1.9 |
| 37 | 2-methylindene | 1266 | 155.7 | 2.6 | - | - |
| 38 | 1-methyl-1H-indrene | 1272 | 21.8 | 0.4 | - | - |
| 39 | 1,2,3,4-tetrahydronaphthalene | 1280 | 51.6 | 0.8 | - | - |
| 40 | 1,2-dihydronaphthalene | 1283 | 25.8 | 0.4 | - | - |
| 41 | 2-ethylindan | 1287 | 9.9 | 0.2 | - | - |
| 42 | 1,4-dimethyl-2- (2-methylpropyl)benzene | 1294 | 30.1 | 0.5 | - | - |
| 43 | naphthalene | 1305 | 77.7 | 1.3 | - | - |
| 44 | $\beta$-citronellol | 1332 | 37.5 | 0.6 | 4.1 | 16.0 |
| 45 | 1-ethyl-1(1-cyclobutylindenethyl) cyclobutane | 1350 | 1.1 | 0.0 | - | |
| 46 | *trans*-geraniol | 1359 | 5.4 | 0.1 | 0.5 | 1.9 |
| 47 | geranial | 1378 | 12.1 | 0.2 | 0.2 | 0.8 |
| 48 | 1H-Indole | 1409 | 3.9 | 0.1 | 0.7 | 2.7 |
| 49 | $\alpha$-4-hydroxy-cyclohexanemethanol | 1449 | 27.2 | 0.4 | 14.0 | 70.4 |
| 50 | *trans*-$\beta$-caryophyllene | 1548 | 2.2 | 0.0 | - | - |
| | **Total** | | **6076.2** | | **25.7** | |

(33.2%) was the most abundant compound as a free form. 2-(2-Hydroxyl-2-propyl)-5-*m*-cyclohexanol and $\alpha$-terpinene-4-ol were the compounds found only in glycosidically bound form of solvent extraction method. Therefore, these two compounds might only be in glycosidically bound form, or they might be formed during acid hydrolysis. The chemical reactions involved in these formations might be the formation of terpene alcohol by acid catalyzed rearrangement of polyol and the oxidation of hydrocarbon terpene resulting in the formation of oxygenated terpene compound (Boulanger and Crouzet, 2000).

For solid phase extraction method using XAD-2 resin, ten glycosidically bound volatile compounds were identified. All of them were also found in free form at lower concentrations. As compared to solvent extraction method, XAD-2 resin extraction method gave less number of types and lower concentrations of glycosidically bound compounds (Figure 2). However, most of glycosidically bound volatile compounds were related to kaffir lime leaf odor. Thus, many studies used XAD-2 only in glycosidically bound volatile compound isolation (Morales and Duque, 2002). The compounds that found in both free and glycosidically bound forms extracted by XAD-2 were hexanal, *trans*-2-hexenal, styrene oxide, 3-diethylbenzene, citronellal, $\beta$-citronellol, *trans*-geraniol, geranial, 1H-Indole, and $\alpha$-4-hydroxy-cyclohexanemethanol.

## CONCLUSION

Solvent extraction method gave the better result of free and glycosidically bound volatile compound than isolation from fresh kaffir lime leaves due to their board ranges of isolated compounds and direct contact of sample and solvent. Free and glycosidically bound volatile compound extracted by solvent method had the kaffir lime leaf characteristic odor. The concentrations of most free and glycosidically bound volatile compounds extracted with XAD-2 resin were lower than these extracted by solvent extraction method, especially the compounds that showed kaffir lime leaf characteristic odor such as citronellal, $\beta$-citronellol, *trans*-geraniol and, *trans*-$\beta$-caryophyllene.

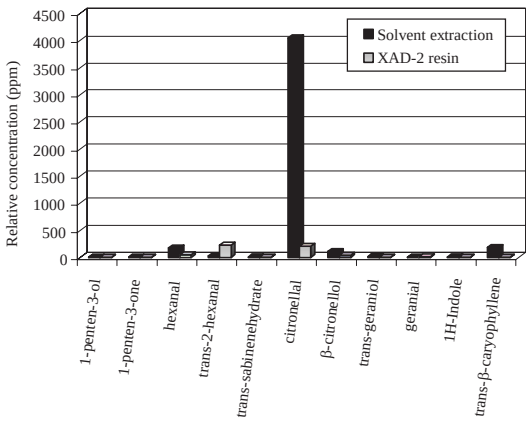


**Figure 1** Free volatile compounds found in both solvent extraction and XAD-2 resin methods.

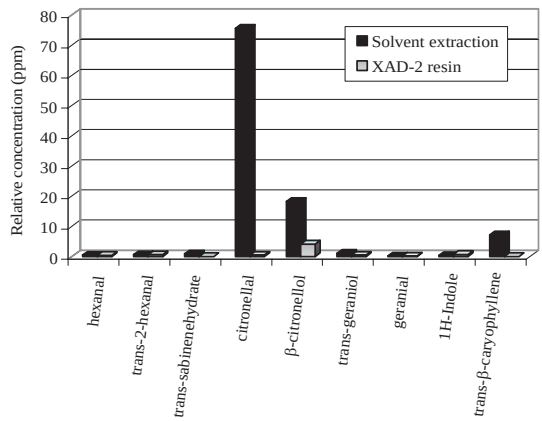


**Figure 2** Glycosidically bound volatile compounds found in both solvent extraction and XAD-2 resin methods

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