JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Application of 2D-HPLC/Taste Dilution Analysis on Taste Compounds in Aniseed (*Pimpinella anisum* L.)

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ABSTRACT: This is the first application of fully automated, preparative, two-dimensional HPLC combined with sensory analysis for taste compound discovery using a sweet and licorice-like bitter-tasting aniseed extract as an example. Compared to the traditional iterative fractionation of food extracts by sensory-guided sequential application of separation techniques, the fully automated 2D-HPLC allowed the comprehensive separation of the aniseed extract into 256 subfractions and reduced the fractionation time from about 1 week to <1day. Using a smart sensory strategy to locate high-impact fractions, e.g., by evaluating first-dimension fractions by reconstituting them from second-dimension subfractions, followed by straightforward application of the taste dilution analysis on the individual second-dimension subfractions revealed the sweet-tasting *trans*-anethole and the bitter-tasting *trans*-pseudoisoeugenol 2-methylbutyrate, showing recognition thresholds of 70 and 68 μmol/L, respectively, as the primary orosensory active compounds in aniseed. 2D-HPLC combined with smart sensory analysis seems to be a promising strategy to speed the discovery of the key players imparting the attractive taste of foods.

KEYWORDS: *two-dimensional liquid chromatography, offline two-dimensional liquid chromatography, LC/×/LC, 2D-HPLC, taste, taste dilution analysis, aniseed*

INTRODUCTION

Flavor perception is caused by the simultaneous stimulation of the various human chemical senses and is triggered by odor- or taste-active chemicals as well as trigeminal stimuli naturally present in food products. Although very important for the overall sensory quality of foods, compared to aroma-active volatiles,[1] still relatively little information is available on key taste compounds.

Driven by the need to discover the key players imparting the attractive taste of foods, the research area of sensomics has made tremendous efforts in recent years to map the sensometabolome and to identify the most intense taste-active molecules in foods using a sequential combination of different chromatographic separation techniques and analytical sensory tools such as the taste dilution analysis[2] (TDA). Examples are the discovery of taste compounds, in black tea infusion,[3,4] cocoa powder,[5,6] roasted coffee,[7,8] whiskey and oak-matured beverage,[9] red currant juice,[10] red wine,[11] cheese,[12] hops and beer,[13,14] black pepper,[15] and asparagus.[16] Moreover, taste modulators have been identified, such as kokumi-enhancing γ-glutamyl peptides in cheese[17] and beans[18] and *N*-(1-methyl-4-oxo-imidazolidin-2-ylidene)-α-amino acids in stewed beef,[19] umami-enhancing (*S*)-morelid in morel mushrooms[20] and N^2-(1-carboxyethyl) guanosine 5′-monophosphate and analogues in yeast extract,[21,22] and, very recently, the allosteric sweet receptor modulator 5-acetoxymethyl-2-furaldehyde generated upon maturation of traditional balsamic vinegar.[23]

Despite this tremendous progress in sensomics-based taste discovery, more efficient high-throughput separation technologies are urgently needed to speed the discovery of taste (modulating) compounds in complex foods and also in plant extracts and biofermentations, respectively. On the analytical scale, two-dimensional liquid chromatography (2D-LC) approaches have been shown to be superior in regard to resolution and separation in the analysis of highly complex samples.[24−26] Having the advantage of shorter analysis times and easy automation, online analytical 2D-LC, with the second-dimension separation taking place simultaneously with the first-dimension separation, has been successfully developed to map biological molecules in complex mixtures.[27,28] In comparison, the collection of effluent fractions of the first dimension prior to commencing the second dimension is known as offline 2D-LC. This technique allows for further treatment of first dimension fractions and offers more flexibility in the design of second-dimension separations. This offers a competitive advantage for preparative 2D-LC separations aiming to collect fractions for another purpose such as biological activity screenings.[29] Fully automated, comprehensive offline two-dimensional separations have been realized by coupling the first-dimension separation with a solid-phase extraction (SPE) device, followed by several secondary separations (HPLC-SPE-HPLC).[30] However, scientific publications employing preparative 2D-LC instruments are comparatively scarce,[31−33] and application of this technique in taste research has not been previously reported.

To introduce a fully automated, preparative HPLC-SPE-HPLC combined with a modified TDA for taste compound discovery in foods, a sweet and licorice-like tasting aniseed extract was used as an example. Due to their pleasant sweet-bitter taste profile and typical odor, the fruits of anise (*Pimpinella anisum* L.) have been used for decades as flavoring for alcoholic beverages such as Greek ouzo, French Pernod, and

Received: June 18, 2014
Revised: August 21, 2014
Accepted: September 4, 2014
Published: September 4, 2014

dx.doi.org/10.1021/jf502896n | J. Agric. Food Chem. 2014, 62, 9239−9245

Turkish raki. Long known as a carminative and expectorant, the essential oil of aniseed exhibits antibacterial and antioxidant properties.[34,35] The odor of aniseed is long-known to be elicited by *trans*-anethole, **1** (Figure 1), the major component of its

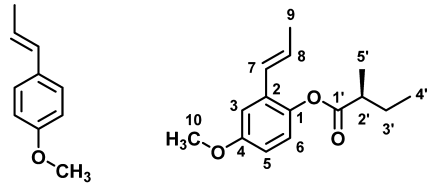

Figure 1. Chemical structures of *trans*-anethole (**1**) and *trans*-pseudoisoeugenyl 2-methylbutyrate (**2**).

essential oil.[36,37] By means of aroma extract dilution analysis and identification and quantitation of most intense odorants, followed by calculation of odor activity values, *trans*-anethole was confirmed to be the character impact compound responsible for the unique aniseed scent.[38] However, systematic study on the oro-sensory activities of aniseed components have not been performed.

Originally invented for the generation of compound libraries for high-throughput screening programs,[39−41] we here report for the first time the application of preparative HPLC-SPE-HPLC combined with a modified TDA for taste compound discovery using a sweet and licorice-like bitter-tasting aniseed extract as an example.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: formic acid, trifluoroacetic acid (Merck, Darmstadt, Germany), anethole, estragole (Sigma-Aldrich, Steinheim, Germany). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). Solvents were of HPLC grade (J. T. Baker, Deventer, The Netherlands) except for *n*-hexane and ethyl acetate (p.A.) (Merck), which were distilled prior to use. Water for HPLC analysis and extractions was obtained from a Milli-Q Advantage A10 high-purity water system (Millipore, Molsheim, France). For sensory analysis, 3 or 5% ethanol (>99.9%) (Merck) was added to Evian bottled water (Danone Waters Deutschland, Frankfurt, Germany). Aniseed was purchased from a local market (Bio Lebensmittel Zander, Mühlheim, Germany).

Sequential Solvent Extraction. After freezing with liquid nitrogen, aniseed was comminuted to a fine powder in an A11 basic batch mill (IKA Werke, Staufen, Germany). An aliquot (240 g) of the ground material was suspended in *n*-hexane (2.5 L) and ultrasonicated for 4 h at room temperature with vigorous stirring. After filtration through a 18.5 cm Schleicher & Schuell filter, the organic solvent was separated from the filtrate under vacuum (40 °C) to afford the solvent extractables. The residue was sequentially extracted with ethyl acetate (2.5 L), followed by methanol (2.5 L) and methanol/water (30:70, v/v; 2.5 L) using the same protocol to afford the *n*-hexane extractables (fraction I, yield = 14%), the ethyl acetate extractables (fraction II, yield = 5%), the methanol extractables (fraction III, yield = 17%), and the methanol/water extractables (fraction IV, yield = 21%); the latter two fractions were separated from solvent under vacuum and, then, freeze-dried twice.

For further separation of fraction I, an aliquot (25 g) was mixed with methanol/water (94:6, v/v; 500 mL) and, after 2 h, the bottom layer separated and again extracted with methanol/water (94:6, v/v; 500 mL). The supernatants were combined and kept at −20 °C until further use. After centrifugation (7000 rpm, 20 min, 0 °C), the liquid layer was separated from solvent under vacuum at 40 °C to afford the sensory active subfraction I-I.

Dry-Injection and 2D-HPLC Separation of Subfraction I-I. An aliquot (1.5 g) of fraction I-I was dissolved in methanol (50 mL), membrane filtered, mixed with Polygoprep 300-50 C4 silica (7 g) (Macherey-Nagel, Düren, Germany) and, after sonication for 20 min, the mixture was separated from solvent under vacuum, thus yielding a free-flowing powder. The stainless steel injection column (20 × 60 mm) was assembled, leaving one side open, and 1 g of blank C4 silica material (1.0 g) was added. After leveling the surface of the stationary phase, the adsorbed fraction I-I was funneled onto the top of the stationary phase, the surface was leveled, the remaining volume of the injection column was filled with blank C4 silica material, the column end fitting was attached, and, then, the injection column was connected to the Sepbox 2D-2000 2D-HPLC system (Sepiatec, Berlin, Germany). Using 0.1% formic acid and 0.02% trifluoroacetic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B, the effluent of the injection column was continuously pumped onto a 150 × 32 mm i.d., 12 μm, VP150/32 Nucleoprep 100-12 C4-end-capped column (Macherey-Nagel) at a flow rate of 40 mL/min. Chromatography was performed with the following gradient: 0 min, 20% B; 6 min, 60% B; 16 min, 100% B; 20 min, 100% B; 21 min, 20% B; 27 min, 20% B. Concomitant with the separation, a flow gradient was run with water, which was mixed with the C4 column effluent using the following flow rates: 0 min, 0 mL/min; 1 min, 50 mL/min; 12 min, 25 mL/min; 16 min, 20 mL/min; 21 min, 20 mL/min; 24 min, 1 mL/min. The effluent was collected in 16 subfractions, namely I-I/1−I-I/16, which were trapped onto VP30/32 Nucleoprep 100-12 C4-end-capped SPE columns preconditioned with water (3 min, 40 mL/min) prior to the separation.

Each subfraction was further separated on a 250 × 21.2 mm, 5 μm, Luna Phenyl-Hexyl column (Phenomenex, Aschaffenburg, Germany) using the following gradient of water (solvent A), methanol (solvent B), and acetonitrile (solvent C) at a flow rate of 20 mL/min: fraction I-I/1, 0 min, A/B/C (83:17:0), 29 min, A/B/C (57:43:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/2, 0 min, 38% B, 29 min, 58% B, 30 min, 100% B, 34 min, 100% B; fraction I-I/3, 0 min, A/B/C (56:44:0), 29 min, A/B/C (37:63:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/4, 0 min, A/B/C (48:52:0), 29 min, A/B/C (23:67:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/5, 0 min, A/B/C (32:68:0), 29 min, A/B/C (28:72:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/6, 0 min, A/B/C (31:69:0), 29 min, A/B/C (20:80:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/7, 0 min, A/B/C (22:78:0), 29 min, A/B/C (13:87:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/8, 0 min, A/B/C (22:78:0), 29 min, A/B/C (9:91:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/9, 0 min, A/B/C (21:79:0), 29 min, A/B/C (9:91:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/10, 0 min, A/B/C (22:88:0), 29 min, A/B/C (7:93:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/11, 0 min, A/B/C (7:93:0), 29 min, A/B/C (5:95:0), 30 min, A/B/C (0:100:0), 34 min, A/B/C (0:100:0); fraction I-I/12, 0 min, A/B/C (3:97:0), 16 min, A/B/C (3:97:0), 17 min, A/B/C (0:100:0), 29 min, A/B/C (0:93:7), 30 min, A/B/C (0:50:50), 34 min, A/B/C (0:50:50); fraction I-I/13, 0 min, A/B/C (3:97:0), 16 min, A/B/C (3:97:0), 17 min, A/B/C (0:100:0), 29 min, A/B/C (0:93:7), 30 min, A/B/C (50:50:0), 34 min, A/B/C (0:50:50); fraction I-I/14, 0 min, A/B/C (0:98:2), 29 min, A/B/C (0:89:11), 30 min, A/B/C (0:50:50), 34 min, A/B/C (0:50:50); fraction I-I/15, 0 min, A/B/C (0:98:2), 29 min, A/B/C (0:80:20), 30 min, A/B/C (0:50:50), 34 min, A/B/C (0:50:50); fraction I-I/16, 0 min, A/B/C (0:100:0), 29 min, A/B/C (0:34:66), 30 min, A/B/C (0:10:90), 34 min, A/B/C (0:10:90). Column equilibration for each separation was 11 min at the initial gradient conditions. Each subfraction, I-I/1−I-I/16, was separated into 16 additional subfractions; for example, fraction I-I/1 was cut into subfractions I-I/1-1−I-I/1-16.

Identification of Key Taste-Active Compounds in 2D-HPLC Subfractions. Aliquots of the first-dimension fractions I-I/1−I-I/16 were reconstituted by combining portions (10 mL) from each of the 16 subfractions collected in the second dimension. The reconstituted

fractions I-I/1−I-I/16 were diluted 1:1 with water and extracted with *n*-hexane (200 mL), and the organic layer was separated from solvent under vacuum at 40 °C and, then, used for sensory analysis. As reconstituted fractions I-I/8 and I-I/9 were judged with the highest sensory impact for sweetness and bitterness, aliquots of each individual of their second-dimension subfractions, namely, I-I/8-1−I-I/8-16 and I-I/9-1−I-I/9-16, were diluted 1:1 with water, extracted with *n*-hexane (50 mL), and, after solvent removal under vacuum (40 °C), the organic residues were kept at −20 °C until further used for sensory analysis. Fraction I-I/8-4, judged with the highest sensory impact, was identified as *trans*-anethole, **1**, by comparing chromatographic (retention time) and spectroscopic data (UV−vis, 1D/2D-NMR) with those of a commercially available reference material. Dried fraction I-I/9-5, judged with high sensory impact, was identified as *trans*-pseudoisoeugenyl 2-methylbutyrate, **2**, by means of LC-TOF-MS and 1D/2D-NMR experiments.

trans-Anethole, ***1***, Figure 1: UV−vis (water/methanol), λ_{max}= 260, 212 nm. NMR data were identical to those of the commercial reference.

trans-Pseudoisoeugenol-2-methylbutyrate, **2**, Figure 1: UV−vis (water/acetonitrile), λ_{max} = 220, 248, 300 nm; UPLC-TOF-MS, *m*/*z* 249.1488 (measured), *m*/*z* 249.1491 (calculated for $[C_{15}H_{20}O_3 + H]^+$); ^{1}H NMR (400 MHz, $CDCl_3$, COSY), δ 6.99 (d, *J* = 3.0 Hz, 1H, [H−C(3)]), 6.88 (d, *J* = 8.8 Hz, 1H, [H−C(6)]), 6.75 (dd, *J* = 8.8, 3.0 Hz, 1H, [H−C(5)]), 6.36 (dd, *J* = 15.8, 1.6 Hz, 1H, [H−C(7)]), 6.20 (dq, *J* = 15.8, 6.5 Hz, 1H, [H−C(8)]), 3.80 (s, 3H, [H−C(10)]), 2.65 (q, *J* = 7.0 Hz, 1H, [H−C(2′)]), 1.92−1.78 (m, 4H, [H−C(3′) and H−C(9)]), 1.63 (ddd, *J* = 13.7, 7.4, 6.4 Hz, 1H, [H−C(3′)]), 1.32 (d, *J* = 7.0 Hz, 3H, [H−C(5′)]), 1.03 (t, *J* = 7.4 Hz, 3H, [H−C(4′)]); ^{13}C NMR (400 MHz, $CDCl_3$, HSQC, HMBC), δ 175.39 (C, [C(1′)]), 157.27 (C, [C(4)]), 141.35 (C, [C(1)]), 131.30 (C, [C(2)]), 128.42 (CH, [C(7)]), 124.49 (CH, [C(8)]), 123.14 (CH, [C(6)]), 113.25 (CH, [C(5)]), 110.98 (CH, [C(3)]), 55.56 (CH3, [C(10)]), 41.23 (CH, [C(2′)]), 29.51, (CH2, [C(3′)]), 18.79 (CH3, [C(9)]), 16.78 (CH3, [C(5′)]), 11.68. (CH3, [C(4′)]).

Sensory Analyses. *Training of the Sensory Panel.* Seven female and six male panelists (25−36 years in age), who had given informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, participated in this study. Each panelist took place in a weekly training session for at least two years to get familiarized with the taste language and methodologies used. Aqueous solutions (2.0 mL; pH 5.9) of the following reference compounds were used for sensory training: sucrose (50 mmol/L) for sweet taste, L-lactic acid (20 mmol/L) for sour taste, NaCl (20 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, monosodium L-glutamate (3 mmol/L) for umami taste, and (+)-catechin (1 mmol/L) for astringency. Sensory analyses were performed by means of the sip-and-spit method in an air-conditioned room at 22−25 °C in three independent sessions. Aimed at preventing cross-model interactions with odorants, nose clips were used by the assessors.

Taste Profile Analysis. Aliquots of fractions I−IV were dissolved in ethanol and made up in water to a maximum content of 5% ethanol. The trained sensory panelists were then asked to judge the intensity of the sweet, sour, umami, salty, bitter, and astringent orosensation on a linear scale from 0 (not detectable) up to 5 (very intense).

Taste Dilution Analysis (TDA). Aliquots of the HPLC fractions were dissolved in "natural" ratios in 10.0 mL of bottled water containing 3% ethanol and sequentially diluted 1:1 with bottled water containing 3% ethanol. The serial dilutions of each of these fractions were then presented in order of increasing concentration to the trained sensory panel, which was asked to evaluate each dilution step by means of a two-alternative forced-choice test. As previously reported, the dilution at which a taste difference between the diluted extract and the blank (control) could just be detected was defined as the taste dilution (TD) factor.[2] The TD factors for each HPLC fraction evaluated by 10 different assessors in three different sessions were averaged. The TD factors between individuals and separate sessions did not differ by more than plus/minus one dilution step.

Two-Dimensional High-Performance Liquid Chromatography System. Preparative 2D-HPLC fractionation of fraction I-I was performed on a Sepbox 2D-2000 HPLC apparatus consisting of a Smartline UV Detector 2500 with a 4061 flow cell (Knaur, Berlin, Germany), a Sedex LT-ELSD detector model 80 (Sedere, Alfortville, France), and two 1800 pumps, one of which was equipped with an A4099-1 ternary low-pressure gradient unit (Knaur), a semipreparative two-channel degasser (Knaur), and a MIKA 3000 mixing chamber (Portman Instruments, Biel-Benken, Switzerland) and was connected to act on the first- and second-dimension flowpath (Figure 2). A self-made mixing chamber consisting of a 20 × 60 mm stainless steel column filled with glass beads (2 mm i.d.) was connected to the first-dimension flowpath (Figure 2). Valve manifolds and fraction collector were custom-made (Sepiatec GmbH, Berlin Germany). Chromatography of the first dimension was conducted using a preparative C4, 150 × 32 mm i.d., 12 μm, Nucleodur column (Macherey-Nagel), equipped with a 2 μm semipreparative frit (IDEX Health and Science, Oak Harbor, WA, USA) and operated at a flow rate of 40.0 mL/min. SPE trapping was facilitated by 16 C4 Nucleoprep VP 30/32 columns (Macherey-Nagel). Chromatography of the second dimension was conducted using a 250 × 21.2 mm i.d., 5 μm, Luna Phenyl-Hexyl column (Phenomenex) with a guard column of the same type at 20 mL/min. For both dimensions, the eluent split ratio was 1:20 (ELSD/UV). Data were acquired using Sepbox Control V2.0.0 software (Sepiatec GmbH, Berlin, Germany), and data analysis was conducted using Sepbox Reporter V2.0.0.3 software.

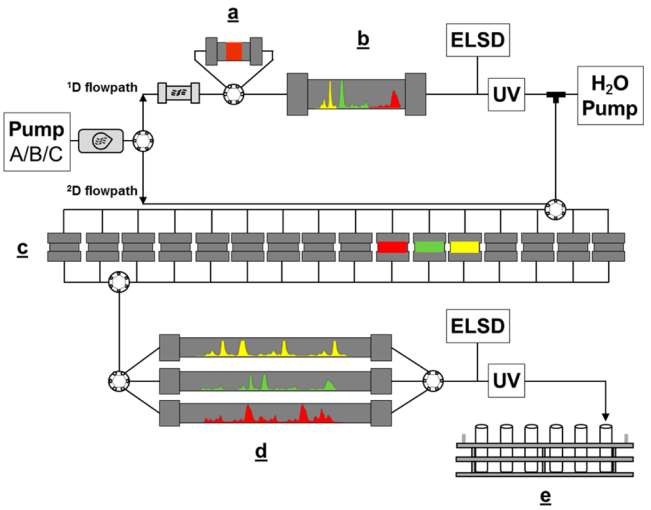

Figure 2. Scheme depicting the principle of operation of the preparative 2D-HPLC system. The sample is introduced using the injection column (a) and subsequently separated on the ^{1}D column (b); fractions are then collected on SPE columns (c), and trapped fractions are further separated on the ^{2}D column (d).

Analytical chromatography of fractions I-I/8-4 and I-I/9-5 was performed on a HPLC apparatus (Jasco, Groß-Umstadt, Germany) consisting of a DG-2080-53 degasser, an LG-2080-02S ternary gradient unit, a PU-2080 Plus pump, an AS-2055 Plus autosampler, an MD-2010 Plus detector with a 6824-H101A cell, and a Sedex LT-ELSD detector model 90 (Sedere). Chromatography was done using an analytical 250 × 4.6 mm i.d., 5 μm, HyperClone ODS 120 column (Phenomenex) operated with a flow rate of 1.0 mL/min. Data acquisition was done by means of Chrompass 1.9.302 (Jasco).

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). High-resolution mass spectra were recorded on a SYNAPT G2S HDMS mass spectrometer (Waters UK Ltd., Manchester, UK) operated in the positive electrospray ionization and high-resolution modus. Sample aliquots (1 μL) in methanol were introduced into the instrument via an Acquity UPLC core system (Waters). The UPLC-TOF-MS system was equipped with a BEH Phenyl, 150 × 2.1 mm i.d., 1.7 μm, column (Waters) equipped with a guard column of the same type and operating at a flow rate of 0.3 mL/min and a temperature of 45 °C. The following gradient was used for chromatography: starting

dx.doi.org/10.1021/jf502896n | J. Agric. Food Chem. 2014, 62, 9239−9245

with a mixture (40:60, v/v) of acetonitrile and aqueous formic acid (0.1% HCOOH), the acetonitrile content was increased to 100% within 3 min and kept at this level for 1 min. All data were lock mass corrected on the pentapeptide leucine enkephalin (Tyr-Gly-Gly-Phe-Leu, m/z 556.2771, $[M + H]^+$) in a solution (2 ng/ μL) of acetonitrile/0.1% formic acid (1:1, v/v). Data acquisition and interpretation were performed by using MassLynx software version 4.1 (Waters) and the tools "isotope model" and "elemental composition".

Nuclear Magnetic Resonance Spectroscopy (NMR). 1D-NMR (^{1}H, ^{13}C) and 2D-NMR experiments (COSY, HSQC, and HMBC) were acquired on a 400 MHz DRX spectrometer (Bruker, Rheinstetten, Germany). Experiments were carried out using the pulse sequences taken from the Bruker software library. Chemical shifts were referenced to TMS. Data processing and interpretation were performed using Topspin version 2.1 (Bruker) and MestRe-Nova version 7.0.2-10008 software (Mestrelab Research, La Coruna, Spain). Samples were analyzed in $CDCl_3$.

RESULTS AND DISCUSSION

To introduce a fully automated, preparative HPLC-SPE-HPLC combined with a modified TDA for taste compound discovery in foods, a sweet and licorice-like tasting aniseed extract was used as an example. Prior to chromatographic separation, first, aniseed fruits were sequentially extracted with solvents of descending hydrophobicity to achieve a crude separation of sensory active components according to their polarity.

Sequential Solvent Extraction and Taste Profile Analysis. Ground aniseed fruits were sequentially extracted with *n*-hexane, ethyl acetate, methanol, and methanol/water (30:70, v/v) to afford fractions I–IV after removal of the solvent under vacuum. Aliquots of fractions I–IV were taken up in water containing 3% aqueous ethanol and were, then, sensorially evaluated by means of taste profile analysis using a linear five-point scale. Sweetness and bitterness were rated with the highest scores of 1.5 and 1.0 in fraction I, whereas fraction III showed the highest impact for astringency judged with a score of 1.2 (Table 1). To preseparate taste-active molecules from triglycerides in the *n*-hexane fraction, an aliquot of fraction I was further separated by extraction with methanol/water (94:6, v/v). The polar extract was separated from solvent under vacuum to afford subfraction I-I showing an increased intensity of sweetness (2.2) and bitterness (1.8) when compared to crude fraction I (Table 1). Therefore, subfraction I-I was used for automated HPLC-SPE-HPLC separation.

Table 1. Taste Profiles of Fractions Isolated from Dried Aniseed Fruits

	fraction[a]				
taste quality	I	II	III	IV	I-I
sweet	1.5	0.8	0.4	0.3	2.2
bitter	1.0	0.8	0.5	0.5	1.8
astringent	1.1	1.2	1.2	1.1	1.4

[a]Fractions isolated from aniseed were sensorially evaluated in 5% aqueous ethanolic solution on a five-point scale from 0 (not detectable) to 5 (intensely perceived) (SD ±0.2).

Setup of the HPLC-SPE-HPLC System. To perform fully automated and comprehensive, offline two-dimensional separations,[42] a Sepbox 2D-2000 was used combining a first-dimension separation with SPE and a series of second-dimension separations (HPLC-SPE-HPLC). The 2D-HPLC instrument comprises two high-pressure pumps, a ternary pump delivering the solvent gradient (A/B/C pump) (Figure 2), and a second pump diluting the effluent of the first dimension with water (H_2O pump) (Figure 2). Flowpath switching was accomplished via electrically actuated Rheodyne valves.

Because extract solubility is not a limiting factor and higher sample loads can be achieved, subfraction I-I was applied onto the column by means of dry-injection. To achieve this, RP4 carrier material was loaded with fraction I-I adsorbed and then placed on top of a layer of blank RP4 material into the injection column (a) (Figure 2) with one endfitting attached. Thereafter, another layer of blank RP4 material was added on top of the sample powder to support the distribution of the mobile phase stream entering the injection column during chromatography.

For chromatography, the mobile phase was delivered by the A/B/C gradient pump and the flow directed through the injection column to elute analytes from the injection column according to their polarity and to subsequently introduce them onto the first-dimension separation column filled with RP4 material (b) (Figure 2). The effluent of the main column was then split (1/20, v/v) between an evaporative light scattering detector (ELSD) and a UV–vis detector for comprehensive recording of eluting analytes. After running through the UV–vis detector, the effluent was diluted with water delivered from the water pump using a T-connector to decrease the acetonitrile content and the solubility of the analytes in the solvent (Figure 2). The effluent, cut into 16 subfractions (I-I/1–I-I/16), was pumped through RP4-end-capped SPE columns to subsequently trap the analytes according to their polarity (c) (Figure 2), whereas the mobile phase was sent to waste. The first dimension concludes with a flushing step of the main separation column, at which time no trap columns were placed in the flow path. To perform chromatography in the second dimension, the individual SPE columns loaded with the analytes of the fractions I-I/1–I-I/16 served as injection columns. One SPE column after the other was placed in the gradient flow path, and analytes are eluted from the trap column onto a second-dimension separation column filled with Phenyl-Hexyl stationary phase (d) (Figure 2). Using a ternary gradient of water, methanol, and acetonitrile, a total of 256 fractions was collected (e), corresponding to 16 fractions collected in the first dimension, each further separated into 16 subfractions, respectively.

Identification and Sensory Analysis of Key Taste Compounds. To effectively trace the compounds imparting the sweet and bitter taste in the 256 subfractions collected by means of 2D-HPLC, aliquots of the second-dimension subfractions corresponding to the same first dimension fractions were recombined to obtain the reconstituted first-dimension fractions I-I/1–I-I/16, and, after dilution with water, the hydrophobic taste compounds were extracted with hexane, the solvent was separated under vacuum, and the residues were taken up in 3% aqueous ethanol and used for taste dilution analysis.[2] To achieve this, the dissolved fractions were stepwise diluted 1:1 with 3% aqueous ethanol and then presented to the trained panelists, who were asked to determine their taste dilution (TD) factors by means of a two-alternative forced-choice procedure. Sweetness and bitterness of reconstituted fractions I-I/8 and I-I/9 were judged with the highest TD factors of 32 and 4, respectively (Figure 3). To trace the key taste molecules, a taste dilution analysis was applied onto aliquots of the corresponding second-dimension subfractions I-I/8-1–I-I/8-9 and I-I/9-1–I-I/9-9, thus showing subfraction I-

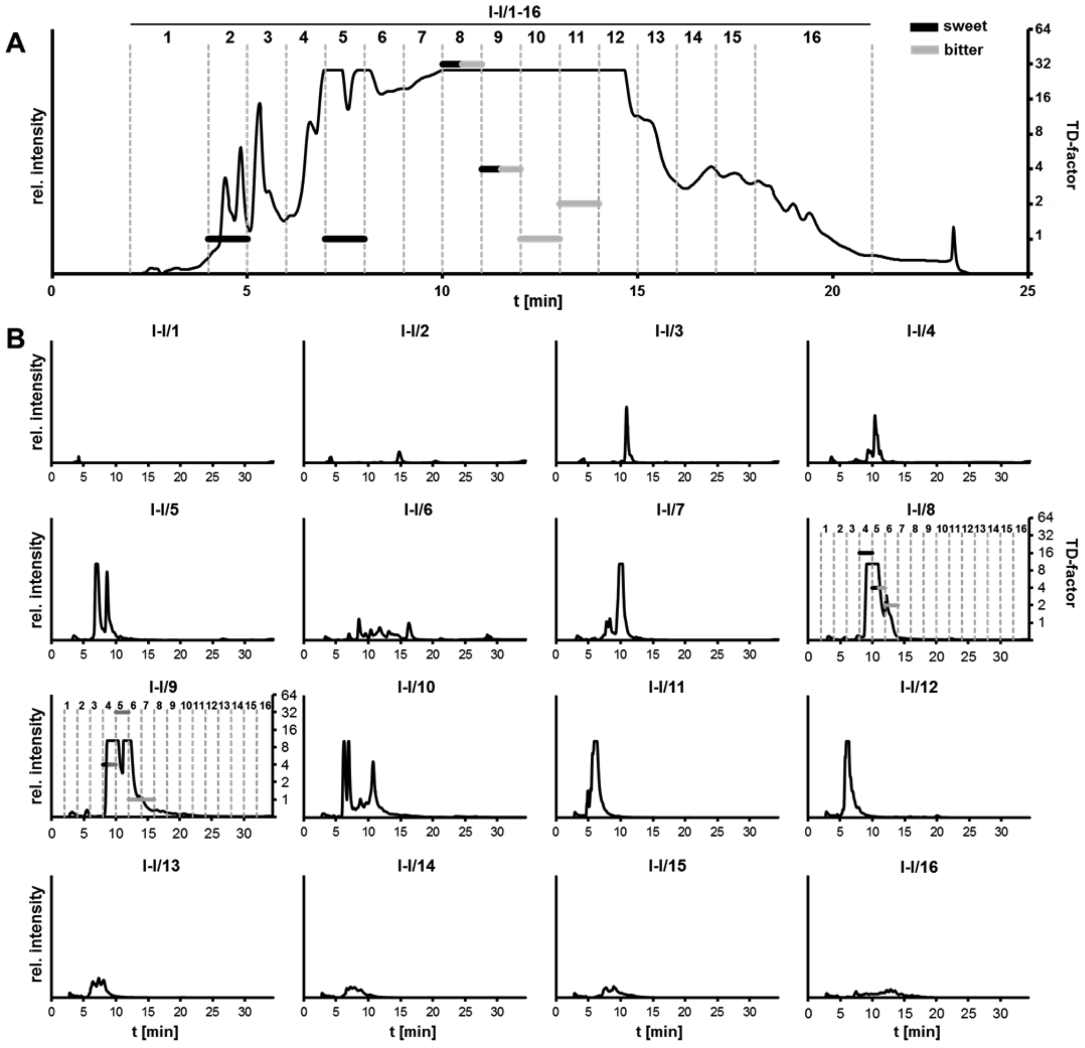

Figure 3. (A) First-dimension separation and taste dilution analysis of fraction I-I isolated from dried aniseed fruits and the corresponding second-dimension separations (B) with taste dilution analyses of the high-impact subfractions I-I/8 and I-I/9.

I/8-4 with the highest TD factor of 16 for licorice-like sweetness and I-I/9-5 with the highest TD factor of 32 for bitter taste, respectively.

Comparison of UV−vis, LC-MS, and 1D/2D-NMR data of the licorice-like sweet-tasting key compound in fraction I-I/8-4 with those found for a commercial reference compound led to the identification of *trans*-anethole (**1**), known to contribute to the typical flavor and sweetish taste of aniseed.[36,37,43,44]

UPLC-TOF-MS(ESI$^+$) analysis of the bitter-tasting key fraction I-I/9-5 revealed m/z 249.1492 as the pseudomolecular ion ($[M + H]^+$), thus suggesting an elemental composition of $C_{15}H_{20}O_3$ for compound **2**. The ^{1}H NMR spectrum of compound **2** showed the aromatic protons H−C(3), H−C(5), and H−C(6) in the chemical shift range between 6.75 and 6.99 ppm with the typical coupling pattern of a 3-fold substituted benzene ring, as well as two *trans*-configured olefinic protons (H−C(7), H−C(8)), resonating at 6.20 and 6.37 ppm with a coupling constant of 15.8 Hz. The remaining 15 aliphatic protons were assigned to the methoxy moiety H−C(10), resonating at 3.8 ppm, the methyl group of the propenyl substituent H−C(9), the terminal methyl protons of the 2-methyl butyrate side chain H−C(4′), the methylene group H−C(3′), the methine proton H−C(2′), and the methylene group H−C(5′) of the methyl branch of the 2-methyl butyrate side chain. Unequivocal assignment of all protons and carbon atoms could be successfully achieved by means of 2D-NMR spectroscopy. For example, the position of the substitutents in the aromatic ring could be assigned by means of an HMBC experiment, which showed a ^3J coupling of the methoxy protons H−C(10) to the quarternary carbon C(4) as well as ^2J and ^3J couplings between the carbon C(2) and H−C(7) at 6.37 ppm and H−C(8) at 6.20 ppm of the *trans*-propenyl substituent. The aliphatic side chain could be assigned by the $^{2,3}J$ C,H correlations of the carbonyl carbon resonating at 175.4 ppm with the protons H−C(2′), H−C(3′) and H−C(5′), respectively (Figure 4). With all spectroscopic data taken into consideration, the structure of bitter compound **2** was determined to be *trans*-pseudoisoeugenol 2-methyl butyrate. Figure 1 shows the (*S*)-enantiomer, which Karl et al.[49] described as the naturally occurring enantiomer. Although this compound has been reported earlier,[45−49] to the best of our knowledge, its bitter activity has not been previously reported in the literature.

To determine the recognition threshold concentration for the sweet taste of *trans*-anethole (**1**) and the bitter taste of pseudoisoeugenol 2-methyl butyrate (**2**), aqueous solutions containing 3% ethanol were evaluated by means of the two-alternative forced-choice test in linear ascending concentra-

dx.doi.org/10.1021/jf502896n | J. Agric. Food Chem. 2014, 62, 9239−9245

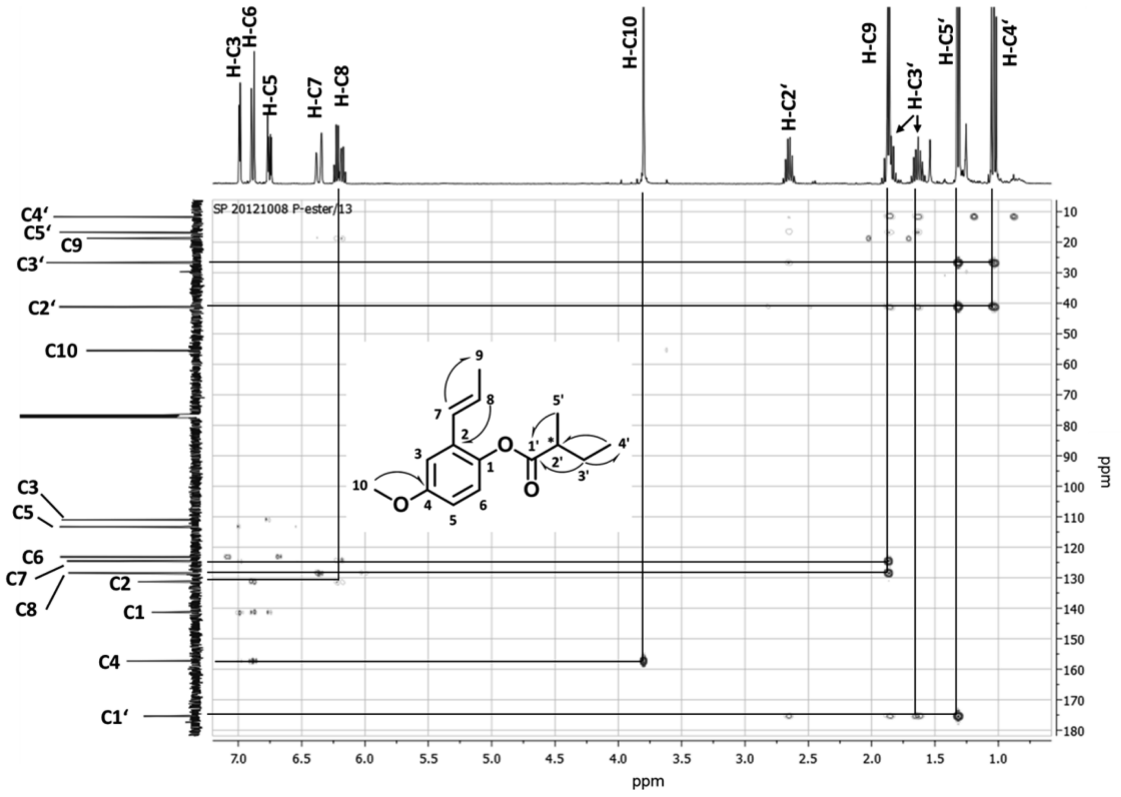

Figure 4. Excerpt of the HMBC spectrum (400 MHz, $CDCl_3$) of *trans*-pseudoisoeugenyl 2-methyl butyrate (**2**).

tions. Anethole was found to have a sweet taste recognition threshold of 70 μmol/L, whereas pseudoisoeugenol 2-methyl butyrate (**2**) showed a bitter recognition threshold of 68 μmol/L.

This is the first report on preparative, two-dimensional HPLC combined with sensory analysis in taste compound analysis. Compared to the traditional iterative fractionation of food extracts by sequential application of separation techniques and sensory analysis (e.g., MPLC → solvent separation → sensory evaluation → HPLC → solvent removal → sensory evaluation, etc.), the fully automated 2D-HPLC offers the possibility to comprehensively separate complex extracts into hundreds of subfractions and to reduce the fractionation time from about 1 week to <1day. Using a smart sensory strategy to locate high-impact fractions, e.g., by evaluating first-dimension fractions by reconstituting them from second-dimension subfractions, followed by straightforward application of the taste dilution analysis on the individual second-dimension subfractions seems to be a promising strategy to speed the discovery of the key players imparting the attractive taste of foods. Application of this technique to more complex food extracts and biofermentations, respectively, is currently in progress to fully evaluate the potential of this 2D-HPLC-Sensomics approach also when combined to cell-based taste receptor assays.

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Notes

The authors declare no competing financial interest.

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