An exploratory study of extractions of celery (*Apium graveolens* L.) waste material as source of bioactive compounds for agricultural applications

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Keywords: Celery; by-products; supercritical fluid; biorefinery; extraction; valorization

Abstract

Interest in the capitalization of waste biomass is steadily increasing in the last years, with many scientists involved in the valorisation of these untapped sources of specialty chemicals and energy, that would, otherwise, be destined to composting and landfills.

In view of developing a circular economy, crop waste is an important resource of specialty chemicals for applications in agriculture. In this study, we extracted celery (*Apium graveolens* L.) waste biomass following a cascade of supercritical fluid extractions with increasing amounts of ethanol as co-solvent. Fractions obtained with this methodology were compared in terms of composition with an extract obtained via Soxhlet extraction employing ethyl acetate, a generally recommended organic solvent with a low toxicity profile. GC-MS analysis revealed the presence of many metabolites with interesting bioactivities. The comparison of the extraction methods shows that the use of hot ethyl acetate results in higher yields than SFE for

selected compounds. Nevertheless, the addition of ethanol as co-solvent can be instrumental for extensively exploiting the waste material by still employing a green technology such as the SFE, also affording fractions with different chemical profiles and thus, different potential applications. Solid residues were subsequently extracted with water to obtain mannitol, a plant osmolyte with biostimulant activity. q^1H-NMR allowed for its quantification in different extracts, confirming celery as an excellent source of mannitol and showing that it's possible to apply water extraction after Soxhlet or SFE to obtain extracts with different potential use destinations. These techniques allowed the identification of possible valorization routes for celery waste as a biostimulant source and crop protection tool.

1. Introduction

Celery (*Apium graveolens* L.) is the most prominent species of its family, the Apiaceae family. It is an annual or biennial plant, widely cultivated in areas with temperate climate as food or for its seeds, which are commonly used as a spice (Malhotra, 2006). It is also largely employed for its essential oils, due to its peculiar aroma profile (Malhotra, 2006). Phthalides have been found responsible for the characteristic odor of celery, in particular 3-butylphthalide, sedanolide and senkyunolide A (sedanenolide) (Uhlig et al., 1987; Oguro and Watanabe, 2011a, 2011b). Celery has been extensively studied in the past years for the isolation and identification of bioactive compounds and for defining its phytochemical profile. The seeds and leaves contain coumarins, furanocoumarins and the corresponding glucosides (Garg et al., 1979, 1980; Ahluwalia et al., 1988), phenolics (Kitajima et al., 2003; Yao et al., 2010; Liu et al., 2017), flavonoids (Mencherini et al., 2007; Zhou et al., 2009; Abdulmanea et al., 2012; Li et al., 2014), and phthalides (Fischer and Gijbels, 1986; Tang et al., 1990; Zhu et al., 2017). Furthermore, it is also of interest to mention that celery is reported as a mannitol-rich vegetable, and has also been used as a model plant for studying the metabolic roles of this widespread sugar alcohol (Stoop et al., 1996). Around 330000 tons of celery were produced in 2022 in the

European Union of which 19000 tons in Flanders (Kips and Van Droogenbroeck, 2014), indicating it is a significant biomass source for chemical extraction and valorization (Eurostat, 2023). Research has been conducted in the past years to valorize the by-products of celery processing, evaluating the possible application of these as flavour source (D'Antuono et al., 2002), as functional food ingredient (Sowbhagya, 2019; Bas-Bellver et al., 2020; Sanahuja et al., 2021; Aşkın Uzel, 2022) or as a source of mannitol and dietary fibers (Rupérez and Toledano, 2003). Mannitol, a C_6 sugar alcohol, is a wide-spread metabolite, found in higher plants, fungi and seaweeds (Grembecka, 2018). It is widely regarded as a compatible solute and an osmoprotectant, providing salinity and drought tolerance in mannitol-producing plants (Stoop et al., 1996). Its exogenous application has already shown beneficial effects on salt-stressed crop plants. Pre-treatment of salt-sensitive wheat seedlings with mannitol (100 mM) exerted alleviative effects upon NaCl treatments, by decreasing lipid peroxidation, enhancing antioxidant enzymes activity and promoting root length (Seckin et al., 2009). Also foliar application (15 or 30 mM) demonstrated beneficial effects on salt-stressed maize, enhancing the biomass production (Kaya et al., 2013).

Supercritical fluid extraction (SFE) employing carbon dioxide is a well-established green technique that owes its attractiveness to the nontoxicity, the ease of removal and the general safety of the solvent. Moreover, the high solvating power and diffusion at moderate temperatures of sc-CO_2, allows the effective extraction of heat-sensitive compounds, preserving their structural features, and hence, their bioactivity (Reverchon and De Marco, 2006; Arumugham et al., 2021). It has been largely explored in the past years for the refining of a wide array of biomasses, from horticultural residues (Benelli et al., 2010) to seaweeds (McElroy et al., 2023) targeting hydrophobic compounds. The addition of co-solvents, such as ethanol, methanol or carboxylic acids among others, gives the advantage of tuning the polarity of the solvent, to obtain compounds of higher polarity (Grigonis et al., 2005; Tzeng et al.,

2007). The step-wise addition of co-solvents allows for a deeper exploration and exploitation of biomass, in a bioprospecting fashion. Despite the high operational costs, the high flexibility and the sustainability of this technique made it our extraction method of choice for exploring celery post-harvest residues. Furthermore, the use of SFE may also represent an interesting way to overcome regulatory limitations regarding the production of plant-derived biostimulants. This concept will be further explained in section 3.3.

SFE has been applied on celery for its essential oils (Sipailiene et al., 2005), and demonstrated its potential in the extraction of bioactive compounds from its seeds (Papamichail et al., 2000, Misic et al., 2020) also from different varieties, such as celeriac (var. *rapaceum*) and wild celery (Järvenpää et al., 1997; Marongiu et al., 2013).

The aim of this study is the exploration of celery waste roots and leaves for their potential application in the agricultural industry. We applied a cascade supercritical CO_2 extraction with the stepwise addition of higher concentrations of ethanol as co-solvent to explore the chemical space of this biomass and evaluate whether separation and enrichment of compounds could be realised via this "green" extraction method. The spent residues were also subsequently extracted with water to maximize mannitol extraction. The obtained fractions are compared with an extract obtained with a traditional method in terms of yield and composition.

2. Materials and methods

2.1. Materials

All chemicals and reagents were commercially available and obtained in analytical purity or higher from Tokyo Chemical Industry Co. and Sigma-Aldrich, except for 3-butylphthalide and sedanolide. 3-butylphthalide was synthesized in-house (procedure available in Supporting Information). Sedanolide was isolated from a hexane extract of the celery waste material (procedure available in Supporting Information). Dried and milled celery (*Apium graveolens*

var. *dulce*) non-marketable portion (leaves and roots) was obtained from Greenyard, a vegetable processing plant located in Flanders, Belgium.

2.2. Soxhlet extraction

Soxhlet extraction was performed on a weighed aliquot of celery waste material. Briefly, the solid plant material was added to a cotton thimble and capped with hydrophilic cotton (previously washed with hexane to remove fat impurities). Ethyl acetate was used as solvent with a S/L (solid to liquid ratio) of 1/30 and the extraction was carried out for 5 hours at reflux temperature. The resulting extract was evaporated *in vacuo* to dryness and weighed (sample "SOX"). The solid celery residue was dried in an oven at 90 °C and subsequently extracted in HPLC grade water at 45 °C for 1 hour, with a S/L of 1/10. The obtained slurry mixture was centrifuged at 4000g for 25 minutes and the supernatant collected, filtered on filter paper and evaporated *in vacuo* to dryness (sample "SOX_H"). In the same way, sample "H2O" was obtained without prior extraction with ethyl acetate.

2.3. Solvent extraction under supercritical and subcritical conditions

An SFE system from JASCO Isogen Life Science was used, containing among others a $scCO_2$ pump with cooler, a co-solvent delivery pump, six parallel flow-through extraction vessels of 10 mL mounted in an oven, a temperature-controlled back-pressure regulator to release pressure and a fraction collector like described by Elst et al. (2018). A schematic representation of the workflow is presented in Figure 1. Powder of dried celery waste material was sieved, and the 250-800 µm fraction was used to fill the 10 mL extraction vessels (about 4.8 g per vessel). The samples were subjected to a cascade of seven subsequent extraction conditions and the extracts were collected in different collections tubes. Step 1 consisted of a supercritical CO_2 extraction at 40°C (20 MPa, 100% CO_2, 60 minutes), with elevation of the temperature in the second step (50°C, 20 MPa, 100% CO_2, 60 minutes). The yield of step 2 was very low.

To avoid inactivation of bioactive compounds, the temperature was not increased above 50°C. IPA (Iso-propyl alcohol) was used as modifier to avoid precipitation of the extracted compounds after the pressure release step. For the next steps, the pressure was increased to 30 MPa (50°C) and cosolvent (ethanol) was dosed at increasing amounts (step 3: 15%; step 4; step 25% and step 5 100%) to increase the hydrophilicity of the extraction solvent. The extraction time of the latter three extraction steps was 120 minutes. Step 6 consisted of a drying step (100% CO_2, 30 MPa, 50°C, 30 minutes) before the extraction residue was removed from the extraction vessel. The solvent was removed by evaporation under a nitrogen gas flow and samples were stored frozen. The water extraction step (step 7) was performed at ambient pressure by bringing aliquots of extraction residue (4 g) in contact with demineralised water for 60 minutes at 45°C (S/L ratio 1/10) on a shaker. The solubilized material was separated from the residue via filtration (25 μm) and the water extract was frozen until further use. The extraction yields calculated were 0.39 +/- 0.12 % (n=4) for step 1 (SFE1); 0.45 +/- 0.08 % (n=3) for step 3 (SFE2), 0.55 +/- 0.02 % (n=3) for step 4 (SFE3); 9.45 +/- 0.98 % (n=3) for step 5 (SFE4) and 32.60 % (n=2) for step 7 (SFE_H).

2.4. Sample preparation and GC-MS analysis

Weighed aliquots (~10 mg) of each sample were suspended in ethyl acetate. In order to increase the homogeneity of the samples, these were sonicated for 10 minutes in an ultrasonic bath. SPE diol cartridges (500 mg / 3 mL, Grace Davison Discovery Sciences) were first conditioned with 9 mL of ethyl acetate. The sample was then loaded and washed with 3 mL of ethyl acetate. The cartridge was further washed with 3 mL of methanol. The ethyl acetate eluates were evaporated to dryness *in vacuo* to be analysed. This procedure was performed in triplicate, affording, for each extract, 3 ethyl acetate eluates (average recoveries from the SPE were: 83% for SFE1,

47% for SFE2, 20% for SFE3, 3% for SFE4 and 60% for SOX). These were derivatized according to Li et al. (2009). Briefly, dry eluate samples were dissolved in acetone to obtain solutions of 7.5 mg/mL. To 300 µL of these solutions, 200 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added and allowed to react for 5 min before analysis. These were performed on an Agilent 8890 GC System equipped with an Agilent J&W HP-5ms (30 m x 0.25 mm x 0.25 µm) column and an Agilent 5977B mass spectrometer with quadrupole mass analyser and Extractor EI source (Electron Ionization, 70 eV). Analysis parameters were as follows: carrier gas helium (1.2 mL/min); split ratio 1/50; inlet temperature 275 °C; oven temperature 60 °C for 1 min, 30 °C/min to 130 °C, hold 2 min, 20 °C/min to 205 °C, hold 1 min, 30 °C/min to 275 °C, hold 15 min; transfer line temperature 280 °C. Background subtraction was performed with the software Enhanced Data Analysis. Qualitative analysis of the chromatograms was carried out with MassHunter Workstation Qualitative Analysis 10.0 (Agilent Technologies). Chromatograms are available in the SI file (SI Fig. 3 to SI Fig. 5). Identification of the compounds was achieved by comparison of the mass spectra with those of reference compounds from the databases NIST98, NIST20 and WILEY6N and with standards, where specified in Table 1. Mass spectra of compounds identified via comparison with database are available in the SI, together with main fragments assignments (SI Fig. 6 to SI Fig. 15).

2.5. GC-MS quantification of metabolites of interest

Quantification was performed by the means of external calibration curves. Acetovanillone was employed as internal standard. Calibration curves were constructed with different concentration ranges for the different metabolites: 1 to 10 µg/mL for scopoletin and tyrosol and 1 to 20 µg/mL for 3-butylphthalide and sedanolide. These metabolites were quantified in the samples eluted from the SPE cartridges with ethyl acetate. A calibration curve with palmitic acid was constructed (5 to 100 µg/mL) with the use of a standard. The metabolite was

quantified in the whole extracts dissolved in acetone and sonicated for 10 minutes. The same procedure was applied for the quantification of succinic acid (with a calibration curve in the range 1 to 15 µg/mL), with the difference that the amount of it in sample SFE3 was quantified employing the ethyl acetate eluate from the SPE procedure. Samples derivatization and GC-MS conditions were as reported in the previous section, and were applied to both the standards for the construction of the calibration curve and the samples. Integration of the peaks was performed with MassHunter Qualitative Analysis 10.0 and achieved by extraction of the ion chromatograms (m/z: 223 and 238 for acetovanillone, 313 for palmitic acid, 247 for succinic acid, 133 and 190 for 3-butylphthalide, 108 for sedanolide, 179 for tyrosol, 264 for scopoletin). Calibration curves construction and calculations were performed with Excel (Microsoft Inc.) while plotting was performed with RStudio (2023.03.0 Build 386).

2.6. ^{1}H NMR quantification of mannitol in water extracts

Extracts were freeze-dried and then weighed aliquots of them were dissolved in 400 µL of D_2O buffered with KH_2PO_4 (90 mM, pH 7, Sigma Aldrich) and 350 µL of a 5 mM DSS solution in D_2O. D_2O and DSS provided a field frequency lock and chemical shift reference (^{1}H δ 0.00 ppm), respectively. Identification of mannitol was performed combining 1D (^{1}H and ^{13}C NMR) and 2D (HSQC) NMR techniques, and comparing the latter with a spectrum available in the public repository HMDB (HMDB00765, https://www.hmdb.ca). Quantification was performed using a standard addition method. Briefly, to 5 different aliquots of 120 µL of each extract, increasing amounts of mannitol (prepared as a 200 µM solution in buffered D_2O) were added. Buffered D_2O was added to obtain a final volume of 520 µL. Each constructed curve was constituted of 5 points (0, 5, 10, 15 and 20 µM). Each sample constituting the curve was analysed in triplicate. NMR experiments were performed using a Bruker AVANCE III

spectrometer, equipped with 1H/BB z-gradient probe (BBO, 5 mm). Spectra were measured at 400 MHz. All spectra were acquired through the standard pulse sequences available in the Bruker pulse program library. Number of scans (NS) was set to 16, while interscan delay was set to 4 s. Spectral data were all processed with Bruker TopSpin version 4.1.3. Exponential window multiplication of the FID, Fourier transformation and phase correction were performed using Bruker AU programs proc_1d. Mannitol quantification was achieved by integration of the signal at 3.85 ppm (2H, *dd*, 11.7, 2.6 Hz) with the reference integration value of the resonance signal at 0.00 ppm of DSS (9H, *s*) (an example of a ^{1}H NMR spectrum used for the quantification can be found in the Supporting Information SI Fig.1). Integrations were performed with Bruker TopSpin version 4.1.3. Standard addition curve construction and calculations were performed using Excel (Microsoft Inc.) while plotting was performed with RStudio (2023.03.0 Build 386).

3. Results and discussion

3.1. Compositional analysis of the extracts

In order to explore the potential products and application of celery waste material, two different extraction methods were applied and compared. Firstly, Soxhlet extraction, a well-established technique that allows for the extensive extraction of metabolites from biological material. Secondly, supercritical fluid extraction, that was applied in cascade, with addition of ethanol as co-solvent to obtain a stepwise increase of polarity, with the aim of obtaining fractions with different chemical profiles in an extraction-fractionation manner. A schematic representation of the workflow is presented in Figure 1.

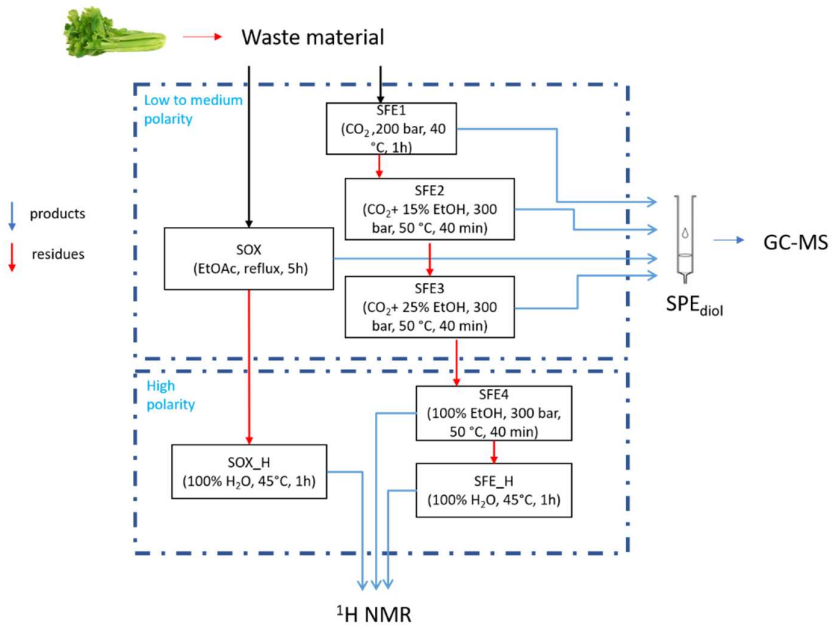

Figure 1 - schematic representation of the extraction and analysis workflow.

To evaluate the composition of the different extracts, a GC-MS method was applied. Despite the long analysis and sample preparation time, GC-MS is a very suitable technique for analysis of complex natural mixtures, in particular in the field of small molecules research. This is due not only to the hyphenation, that allows for separation of metabolites, but mainly to the standard electron ionization (at 70 eV), that allows fragmentation patterns comparison with largely available databases. This enhances dereplication and tentative identification of compounds. Furthermore, in order to overcome the limited range of analysable metabolites, many derivatization techniques were developed in the recent years (Moldoveanu and David, 2018). In our work, derivatization of the samples was performed according to Li et al. (2009), which demonstrated a fast derivatization method for phenolic compounds based on the use of acetone as a solvent and BSTFA as a derivatizing agent. This method was applied to all the extracts from the celery waste material. At first, the GC-MS analyses revealed very complex total ion chromatograms. Database search of the mass spectra of the most prominent peaks indicated a large presence of carbohydrates. As the scope of the analysis was profiling the extracts in terms

of medium to low polarity compounds, an SPE diol filtration was performed before the injection in the GC system. As reported on manufacturers' websites and application notebooks, diol modified silica is commonly used for the retention of polar compounds in normal phase mode, due to the free hydroxy moieties (Thermo Scientific, 2011; Deflaoui et al., 2021). The percentage recoveries from the SPE-diol cartridge with ethyl acetate were inversely proportional to the amount of EtOH added during the extraction step. This is probably due to a higher amount of carbohydrates co-extracted. The extraction or fractionation of carbohydrates with $scCO_2$ with the addition of polar modifiers, although not common, was reviewed (Mena-García et al., 2019; Lefebvre et al., 2021). This hypothesis was also strengthened by the GC-MS analysis of the methanol eluates (with the same derivatization method as for the ethyl acetate eluates), in which most of the peaks were tentatively identified as monosaccharides (data not shown). For SFE3 (obtained with 25% of ethanol) the amount of the ethyl acetate eluate was very low, resulting in a poorly resolved GC-MS spectrum. For this reason sample SFE3 was excluded from the qualitative evaluation. Nevertheless, this was used for absolute quantification of compounds of interest (section 3.2), as for this application, Extracted Ion Chromatograms (EIC) were used. Results of the GC-MS analysis are reported in Table 1, expressed as relative area percentage.

Table 1 - Compounds identified via GC-MS analysis by either comparison with database (with percentage match with database spectra) or with standards ("Std"). Relative quantities are expressed as of the total peak area. Compounds that were further quantified, due to their interesting potential applications are indicated in bold.

Compound	RT (min)	Score (% match)	SFE1 (100% CO_2)	SFE2 (15% EtOH)	SOX
Organic acids					
Lactic acid, 2TMS	4,141	Std	0,3	0,5	1,1
Hexanoic acid, TMS	4,214	Std	0,3	0,3	18,1
Ethyl(trimethylsilyl) succinate	5,994	99		2,4	
Octanoic acid, TMS	6,161	Std			1,0
Succinic acid, 2TMS	**6,715**	**Std**	**8,9**	**21,8**	**24,6**
Fumaric acid, 2TMS	7,028	Std		1,0	0,4

Nonanoic acid, TMS	7,148	Std			0,3
Malic acid, 3TMS	8,343	Std		0,8	
4-Hydroxynon-2-enoic acid, 2TMS	9,1	95	2,2	2,6	1,0
Azelaic acid, 2TMS	10,597	Std	1,0	3,0	0,9
Myristic acid, TMS	10,919	95	0,4		
4-Coumaric acid, 2TMS	11,523	99		1,0	
Ethyl palmitate	11,747	99		1,6	
Palmitic Acid, TMS	**12,005**	**Std**	**33,9**	**17,6**	**24,1**
Ferulic acid, 2TMS	12,272	95		0,4	
Linoleic acid, TMS	12,745	99		1,4	
Stearic acid, TMS	12,855	Std	1,3	1,8	1,6
Aldehydes					
2-Decenal	6,177	Std	1,0		
2,4-Decadienal (*isomer 1)*	6,528	Std	0,2		
2,4-Decadienal (*isomer 2)*	6,765	Std	0,5		0,2
Phthalides					
3-butylphthalide	**9,616**	**Std**	**4,3**	**0,7**	**4,8**
Sedanolide	**10,229**	**Std**	**1,1**	**0,6**	**1,0**
Phenols and coumarins					
Tyrosol, 2TMS	**8,944**	**Std**	**1,0**	**1,9**	**1,3**
Scopoletin, TMS	**12,081**	**Std**	**0,2**	**0,5**	**0,3**
Xanthotoxin	12,128	97	0,5	0,3	0,4
Bergapten	12,234	97	0,5	0,3	0,4
Isopimpinellin	13,016	90		0,2	
Alcohols					
Glycerol, 3TMS	6,342	Std	0,6	2,2	0,2
Triethylene glycol, 2TMS	8,424	Std		3,6	
1-Hexadecanol, TMS	11,57	Std			2,5
1-Octadecanol, TMS	12,487	Std			1,3
Sterols					
Stigmasterol, TMS	25,265	Std	21,2	5,4	5,2
β-sitosterol, TMS	26,945	98	12,6	5,7	2,6
Others					
Erythrono-1,4-lactone, 2TMS	7,376	83		0,5	0,7
Dibutyl phthalate	11,646	Std			0,8
unidentified			8	21,9	5,3
Total area			1.96E+08	2.56E+08	1.99E+08

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255 As depicted by the table, SFE1, obtained with 100% supercritical CO_2, is, as expected,
256 relatively enriched of highly hydrophobic and volatile compounds from the celery waste
257 material, of which some already described in literature, such as palmitic acid, C_{10} unsaturated

aldehydes, stigmasterol and β-sitosterol, among others (Daukšas et al., 2002; Sipailiene et al., 2005). Stigmasterol and β-sitosterol are well known added-value phytosterols with multiple health related beneficial activities and numerous potential applications in food fortification (Poudel et al., 2023). Furthermore, β-sitosterol was identified as the main active compound in an acaricidal extract from *Lactuca sativa* (Li et al., 2018). Two aldehydes, 2-decenal and 2,4-decadienal, that are relatively enriched in fraction SFE1, have been extensively studied for their activity on the root knot nematode *Meloidogyne incognita*, and their egg hatch inhibition and biological cycle arrest effects demonstrated both as single compounds and as a mixture, depicting a strong synergistic activity at ppm concentrations (Ntalli et al., 2016). The addition of ethanol as co-solvent increases the polarity of the extracting mixture, generating more polar fractions, as depicted by the higher relative abundance of succinic acid in SFE2. Some compounds are spread among different fractions of the cascade extraction, showing that the use of pure CO_2 is not sufficient for their extraction under the applied conditions. This behaviour is further discussed in section 3.2, where the absolute quantification is performed.

Some of the metabolites that are present in the different extracts and fractions have been tested by researchers in the past for different applications related to crop protection and biostimulation. These bioactivities are summarised in Table 2.

Table 2 - Selected metabolites present in the different extracts and fractions and their agriculture related bioactivities reported in literature.

Compound	Bioactivity	**Mode of action**	Literature source
Palmitic acid	Acaricidal	Mortality	Liu et al., 2019
	Insecticidal	Larval viability reduction	Pérez-Gutiérrez et al., 2011
	Antifungal	Spore germination inhibition	Liu et al., 2008
	Herbicidal	Germination reduction and seedling growth inhibition	Shen et al., 2022

Succinic acid	Growth and defence mechanisms stimulant	Energy metabolism regulation	Morgunov et al., 2017
	Antibacterial	Growth inhibition	“
	Antifungal	“	“
	Nematicidal	Mortality	“
Sedanolide	Mosquitocidal	Larvae mortality	Momin and Nair, 2001
	Antifungal	Growth inhibition	“
	Nematicidal	Mortality	“
3-butylphthalide	Herbicidal	Shoots growth reduction, germination delay	Sbai et al., 2017
	Insecticidal	Larvicidal and adulticidal activity, acetylcholinesterase inhibitory activity	Tsukamoto et al., 2005
Scopoletin	Antifungal	Germ tube elongation and conidium germination inhibition, fungicidal	Gnonlonfin et al., 2012
	Acaricidal	AcChE, Na^{+} -K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase inhibition	Ma et al., 2020; Liu et al., 2023
Tyrosol	Visible light enhanced phytotoxic	Leaf necrosis upon puncture	Zatout et al., 2021

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278 *3.2. GC-MS quantification of metabolites of interest*

279 In view of the many interesting potential applications in the agricultural field of the
280 beforementioned metabolites, an absolute quantification was carried out, and their yields in the
281 different extracts were calculated. As mentioned before, the samples were subjected to SPE
282 diol treatment and, for the qualitative analysis of the extract, the ethyl acetate eluates were
283 analysed. The analysis of the subsequent methanol eluates revealed that palmitic acid and
284 succinic acid were the only analytes which were partially retained on the cartridge, thus eluting
285 with both solvents. For this reason, their quantification was carried out by derivatizing and

analysing the whole extracts. In Table 3 yields of the different extracts and concentrations of the analytes in these extracts are reported. As depicted by the table, extracts obtained with pure supercritical carbon dioxide and those obtained with Soxhlet extraction, contained comparable amounts of the more apolar compounds. Furthermore, in the case of the phthalides, higher concentrations are obtained with supercritical CO_2, although this trend is not statistically supported. For these compounds it's also clear that the second SFE step, with addition of the co-solvent is beneficial, but to a lesser extent than for the other compounds. In fact it is evident that for some compounds, the sole extraction with 100% CO_2 was not sufficient for their exhaustive recovery from the waste material as the compounds were also present in the subsequent extracts. This, in particular, applies to palmitic acid, that is the most abundant compound, and to the more polar compounds succinic acid, scopoletin and tyrosol.

Table 3 – Yields of extractions (expressed as weight percentage) and compounds concentration in the analysed samples (expressed as mg/g of extract ± sd, n=3).

Sample	Yield (% wt.)	Palmitic acid	Succinic acid	3-Butylphthalide	Sedanolide	Scopoletin	Tyrosol
SOX	1.78	18.59 ± 4.21	6.16 ± 0.49	2.77 ± 0.34	4.77 ± 0.55	0.66 ± 0.08	0.46 ± 0.04
SFE1 (100% CO_2)	0.39	18.27 ± 2.23	2.77 ± 0.42	3.61 ± 0.57	6.77 ± 0.98	0.57 ± 0.10	0.47 ± 0.12
SFE2 (15% EtOH)	0.45	7.88 ± 0.66	4.22 ± 0.29	0.55 ± 0.18	1.35 ± 0.26	0.59 ± 0.06	0.46 ± 0.05
SFE3 (25% EtOH)	0.55	6.36 ± 0.57	1.47 ± 0.97[a,b]	0.13 ± 0.07	0.31 ± 0.17	0.21 ± 0.12	0.11 ± 0.04

[a]n=2; [b] Quantified in the SPE-EtOAc eluate

This behaviour is in accordance with previous reports of supercritical fluid extraction of scopoletin, in which it was demonstrated that the addition of a co-solvent (ethanol, in the specific case), was instrumental for the extraction of the metabolite, due to its higher solubility in a more polar medium (Sajfrtová et al., 2005). This may also apply to succinic acid and to the polar biophenol tyrosol. It is also evident from the table that traditional Soxhlet extraction

with ethyl acetate gave a much higher yield of whole extract and, therefore, also much higher yields of the metabolites of interest, as shown in Figure 2.

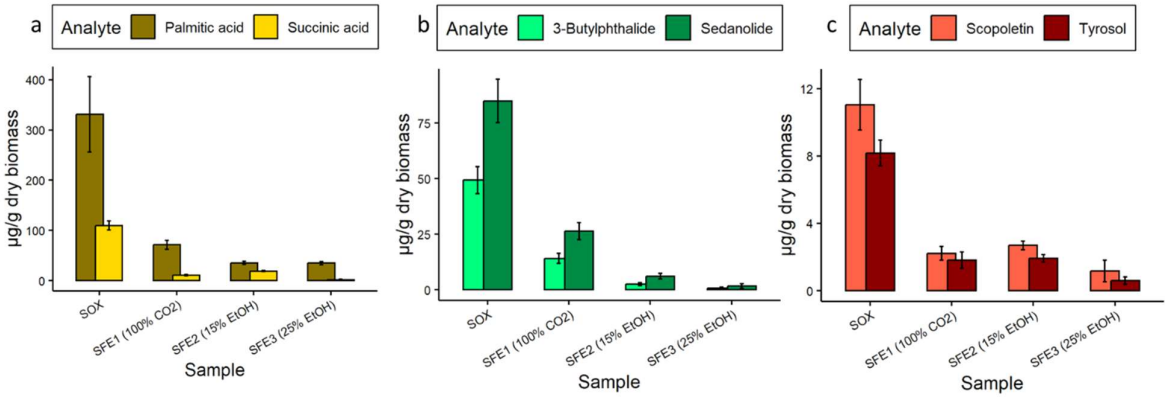

Figure 2 - from left to right: a) yield of palmitic and succinic acid; b) yield of phthalides; c) yield of scopoletin and tyrosol. All yields are expressed as µg/g of dry waste material. Error bars represent standard deviation.

In Figure 2c, the yields of polar scopoletin and tyrosol in the different fractions are compared, depicting that the addition of 15% of ethanol allows for a more exhaustive extraction of both compounds. This applies also to succinic acid, as depicted in Figure 2a. Nevertheless, the traditional Soxhlet extraction performs better, as depicted in Table 4, in which sums of yields of the different compounds, extracted with the cascade supercritical fluid extraction (SFE = SFE1 + SFE2 + SFE3), are compared to the yields obtained via Soxhlet extraction with ethyl acetate. In this way, it was possible to calculate percentage recoveries obtained via the cascade supercritical fluid extraction relative to the Soxhlet extraction, considered the most exhaustive method.

Table 4 - Percentage recoveries of the cascade supercritical fluid extraction relative to the Soxhlet extraction and estimated yields (expressed as µg/g of dry biomass.

Compound	% recovery	Yield (SFE) µg/g dry biomass

Palmitic acid	42.8	141.7
Succinic acid	29.4	32.2
3-Butylphthalide	35.0	17.3
Sedanolide	40.2	34.2
Scopoletin	55.2	6.9
Tyrosol	53.0	4.3

322

323

324 The main difference between the extraction methods can be observed in the total yields of crude
325 extracts (Table 3). Although the concentrations of metabolites are similar in SOX and in SFE1,
326 the lower yield of SFE1 gives lower yields of the target metabolites. Optimization of the
327 cascade supercritical fluid extraction could help to increase its yield and, consequently, the
328 total recovery of these metabolites of interest, still employing this recognized safe and
329 sustainable extraction technique. Pressure fluctuation was already proven a successful
330 technique for increasing the extraction rate from a similar feedstock, with little to no effect on
331 the chemical composition of the resulting extract (Daukšas et al., 2002). Employment of
332 optimization techniques, such as response surface methodology (RSM), varying pressure and
333 temperature can also allow tailoring of the process for a specific material to obtain higher yields
334 (Kessler et al., 2023). Optimization of the cascade steps could afford extracts enriched with
335 different classes of metabolites.

336 The results also suggest that ethyl acetate may have valuable properties for increasing the
337 efficiency of biomass extraction. Despite the high chemical waste burden associated with
338 organic solvents, ethyl acetate figures as a "recommended solvent" in the CHEM21 solvent
339 selection guide (Prat et al., 2015; Funari et al., 2023). This is due to the low toxicity profile and
340 the low environmental impact. Its moderate boiling point also allows for low-energy-
341 demanding evaporation and subsequent recovery of the solvent. The use of this solvent in green

342 extraction techniques, such as microwave assisted extraction, accelerated solvent extraction or
343 gas expanded liquid extraction could be instrumental for the valorisation of this wide-spread
344 waste material. Nevertheless, the employment of organic solvents in a biorefinery inspired
345 process, may represent a limitation in some current regulatory framework, whereas the
346 employment of supercritical CO_2, on the other hand, can be beneficial. This will be further
347 explained in section 3.3.

348 The evolutionary cohabitation of plants and pests is a driver for the development of resistance
349 from either side and has led to the development of a wide range of plant metabolites with
350 pesticidal activity. The combination of different bioactive compounds in plant extract-derived
351 biopesticides offers the advantage of combatting pests and diseases through various
352 mechanisms (Ayilara et al., 2023). We detected several molecules with pesticidal activity in
353 celery waste suggesting that the extracts are a potential source for creating new crop protection
354 products. For instance, the fractions SFE1 and SOX contained scopoletin, palmitic acid, β-
355 sitosterol and stigmasterol, molecules for which acaricidal activity has been reported (Cheng
356 et al., 2012; Li et al., 2018; Liu et al., 2019; Ma et al., 2020). These molecules may have
357 synergistic or additive activity against mites and specific combinations may be lethal to the
358 insects even when applied at sublethal doses (Pavela, 2014; Tak and Isman, 2017). A similar
359 kind of cooperation has been reported for combinations of botanicals and microbial
360 biopesticides (Reddy and Chowdary, 2021). Further research is needed to determine the
361 activity of the acaricidal compounds found in the celery waste, and whether specific
362 combinations enhance the bioactivity.

363 *3.3. ^{1}H NMR quantification of mannitol in water extracts*

364

Mannitol was first identified by comparison of the HSQC spectrum (SI Fig.2) with a reference spectrum retrieved from the public repository HMDB. As expected, it is the main metabolite in the water extracts, and its resonance signals are the most prominent in all the ^{1}H NMR spectra (an illustrative spectrum is available in the Supporting Information SI Fig.1). The different extracts were evaluated in terms of yield and mannitol content based on ^{1}H NMR quantification. This technique is characterised by very short analysis time, with limited or no sample preparation. Nevertheless, high operational costs, absence of a chromatographic separation (unless in hyphenated setups) and low sensitivity, make it not suitable for all applications. In our work, being mannitol the main component of the mixture, with distinguishable and characteristic peaks, q^{1}H-NMR was the most straightforward technique for its quantification, especially in combination with a standard addition quantification method.

As shown in Table 5, the highest yields are obtained by direct extraction with water and by extraction with water after Soxhlet extraction, with a negligible difference. Nevertheless, by summing up yields obtained by ethanol and water extractions after SFE, approximately the same results are obtained, showing the limited influence of the previous processing steps on the yield of polar extracts. Fraction SFE4 was generated with bioprospecting purposes, with the aim of obtaining an extract of medium to high polarity. Nevertheless, ^{1}H-NMR analysis revealed its composition as very similar to the one of the subsequent aqueous extracts SFE_H. The higher yield of SFE_H demonstrates that using ethanol alone is not sufficient for obtaining an exhaustive extraction of mannitol, probably due to the scarce solubility of this compound in alcohols. This, and the very low recovery obtained from SPE diol filtration of sample SFE4, demonstrate the limited value of this extraction step. By considering the yields and the mannitol contents in the different extracts, we calculated mannitol yields (Figure 3). The results corroborate with the higher yield of mannitol using water and shows that the ethyl acetate extraction step has very limited influence on the yield of mannitol.

Table 5 - Yields and mannitol content of the different polar extracts. SOX_H is the water extract of the residue of the Soxhlet extraction. SFE_4 and SFE_H are the ethanol and water extracts of the residue of the cascade SFE extraction, respectively.

Sample	Yield (% wt)	Mannitol content (mg/g extract)
H2O	44.6	333.6
SOX_H	44.5	326.2
SFE_4	9.4	284.3
SFE_H	32.3	291.5

As demonstrated by Rupérez and Toledano (2003), celery by-products are a good source of low molecular weight carbohydrates (LMWC), with mannitol being the most abundant (13 – 15% dry weight). They also demonstrated that stalks are the most promising part in terms of LMWC and mannitol content. In the present work, only waste leaves and roots were subjected to extraction.

The viability of water extraction of mannitol has been demonstrated from different plant species and with different techniques. Ghoreishi and Shahrestani (2009) employed subcritical water to extract mannitol from olive leaves, obtaining an optimum yield of around 6.14% (wt.) (76.75% wt. of the 8% mannitol weight, as stated in the publication). McElroy et al. (2023) applied microwave assisted extraction with water as solvent to extract mannitol from *Saccharina latissima,* obtaining a yield of 3.6% (wt. mannitol/wt. dry biomass). In this study, a mannitol yield of 15% (wt. mannitol/wt. dry biomass) is obtained, confirming celery waste material as a rich source of this sugar alcohol, not only in the stalks, as previously described (Rupérez and Toledano, 2003), but also in the roots and leaves, which are normally discarded.

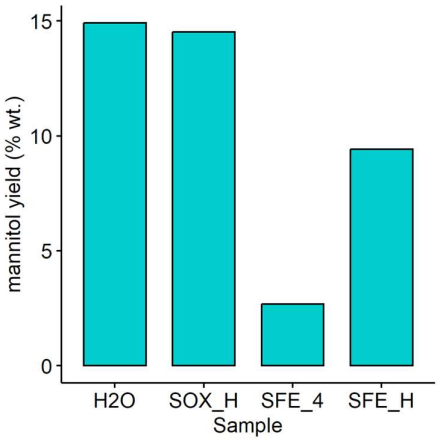

Figure 3 - Mannitol yields (expressed as % wt.). H2O is the water extract of the celery waste material. SOX_H is the water extract of the residue of the Soxhlet extraction. SFE_4 and SFE_H are the ethanol and water extracts of the residue of the cascade SFE extraction, respectively.

Furthermore, these results show the possibility to obtain from one feedstock, through different subsequent processes, a diverse set of extracts, with different potential applications in the field of agriculture, in a phase II biorefinery fashion (Clark and Deswarte, 2015).

In the development of a plant protection product, legislation and regulation need to be taken into account. In Europe, for example, while for the authorization of a botanical biopesticide (that falls under the definition of "active substance", regulated by the European Commission (2013) (Karamaouna et al., 2023)), there are no particular limitations in the production process of the active substance, the regulation for biostimulant approval is more stringent. As regulated by the European Commission (2019), an EU approved biostimulant may contain plant extracts or parts, but these can only undergo physical processing and/or water or $scCO_2$ extraction. For this reason, optimization of a SFE involving the use of solely CO_2 would be instrumental for developing a circular bioeconomy-oriented process, not only for increasing its efficiency and yield, but also for overcoming regulatory limitations.

4. Conclusions

With the aim of exploring possible valorisation strategies for celery waste material, two different extraction approaches were evaluated. Cascade supercritical fluid extraction, with the use of increasing amounts of ethanol, was compared to a traditional Soxhlet extraction with ethyl acetate. The resulting extracts were analysed via GC-MS, which revealed the presence of secondary metabolites with interesting bioactivities. Stepwise increase of polarity, obtained with the addition of ethanol as co-solvent, generates fractions with different chemical profiles. The process could be further tailored to obtain better separation and thus fractions with distinct bioactivities and potential applications. Phthalides (sedanolide and 3-butylphthalide), phenolics (scopoletin and tyrosol) and organic acids (palmitic and succinic acid) were quantified in different celery extracts. In term of yields, the employment of hot ethyl acetate gave better results compared to the supercritical fluid extractions that were applied (without optimization towards yields). Subsequently extracting the solid residues with ethanol and/or water, affords extracts rich in mannitol, a known plant osmoregulant, which is the most abundant extractable metabolite (15% wt./wt. dry biomass) demonstrating the possibility to use these two techniques in series, in a biorefinery fashion. Our results demonstrate that celery waste material contains a diverse set of metabolites that can be extracted using eco-friendly methods, and creates new opportunities to explore potential valorization of this by-product in a circular bioeconomy fashion.

5. Acknowledgments

Kathy Elst (VITO) and Peter De Rechter (VITO) are acknowledged for their contribution to the SFE analyses, Queenie Simons (VITO) for the water extraction.

6. Funding

This research was funded by Fonds Wetenschappelijk Onderzoek (FWO)—Strategic Basic research (SBO), grant number S006017N and Ghent University through a BOF-BAS grant (01B02720) and project (BOF23/CDV/153).

7. Author contributions

Conceptualization: P.M., L.B., S.M.; methodology: P.M., L.B.; formal analysis: P.M.; resources: D.G., S.M.; writing – original draft preparation: P.M.; writing – review & editing: L.B., D.G., S.M.; funding acquisition: D.G., S.M., L.B.

8. Competing interests

The authors declare no competing interests.

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Supporting information

An exploratory study of extractions of celery (*Apium graveolens* L.) waste material as source of bioactive compounds for agricultural applications

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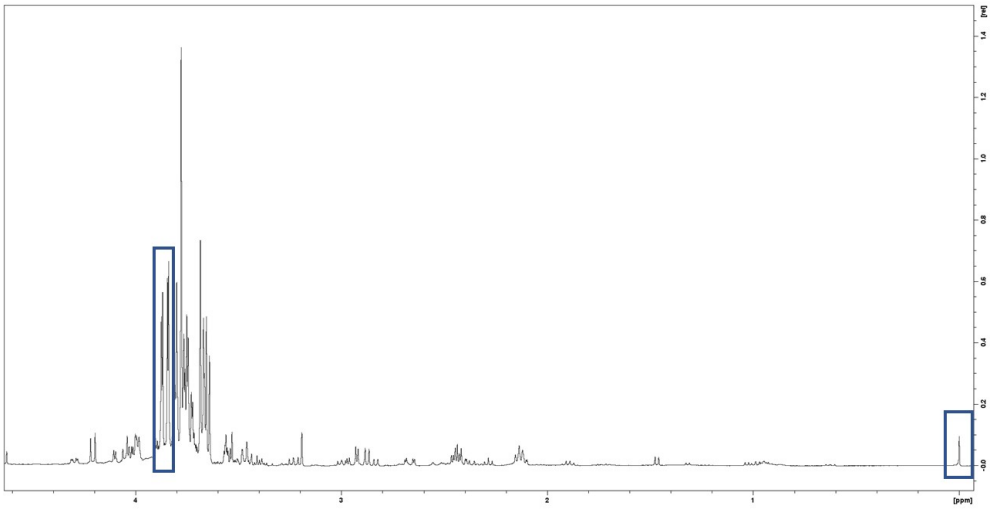
Synthesis of 3-butylphthalide

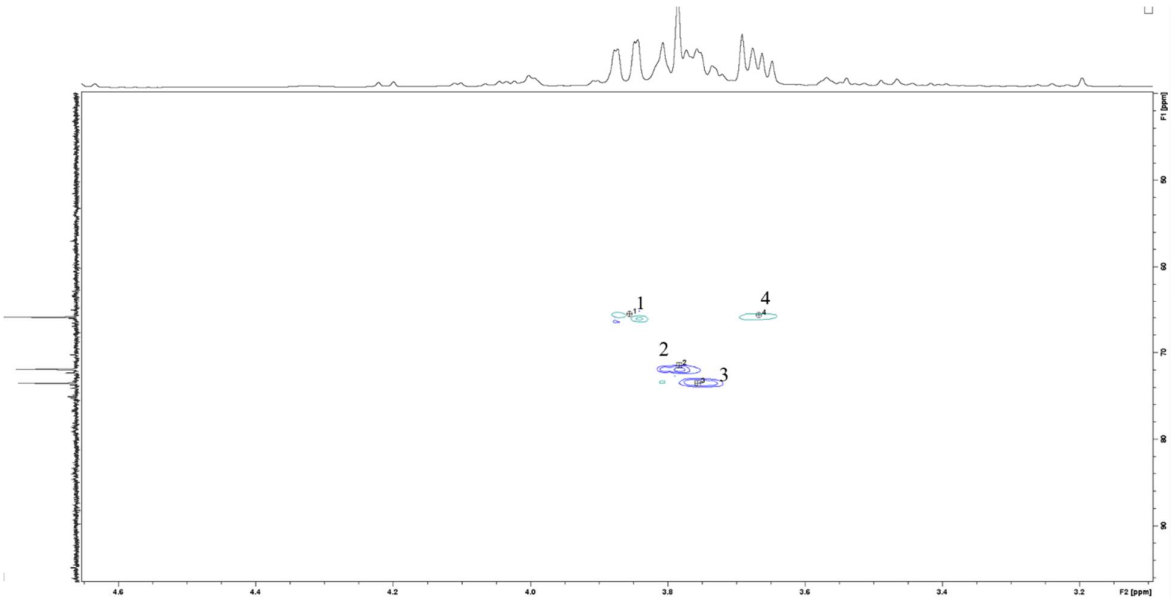
The compound was synthesized according to (Desi Reddy and Padaki, 2020) with some modifications. Briefly, the Grignard reagent was prepared from 500 mg of Mg flakes in 6 mL of THF. To this 2 mL of butyl chloride (19 mmol) in 4.6 mL of THF were slowly added under inert atmosphere. The reaction mixture was stirred at room temperature (occasionally warmed up with external hot air) until most of the Mg flakes were disappeared. 1 g of 2-carboxybenzaldehyde (6.7 mmol) were added to the obtained Grignard reagent, and the reaction mixture was stirred at 30 °C overnight. In order to quench the reaction, 4 mL of a saturated solution of NH_4Cl were slowly added. Afterwards, 4 mL of HCl (18%) were added and the reaction was left under vigorous stirring for 3 h. The mixture was extracted 3 times with 10 mL of ethyl acetate and the organic layers were collected together and washed with 10 mL of a saturated $NaHCO_3$ solution twice. This was then dried over $MgSO_4$ and evaporated *in vacuo*. Purification of the 3-butylphthalide was achieved using reverse phase automatic flash chromatography (Grace Reveleris flash chromatography system) employing a H_2O/acetonitrile gradient. The isolated fraction was evaporated *in vacuo*, then dissolved in diethyl ether with the addition of concentrated HCl, in order to remove the impurities. After 3 h of stirring at room temperature, water was added, and the organic layer collected. The water phase was washed with hexane to recover the product. The two organic phases were collected together, dried over $MgSO_4$, filtered and evaporated *in vacuo*. This afforded pure 3-butylphthalide, whose identity was confirmed via ^1H and ^{13}C NMR, and LC-ESI-MS analysis with literature data comparison (Sbai et al., 2017).

Isolation of sedanolide

Crude celery material was extracted with hexane, with the use of a Soxhlet apparatus (S/L 1:30, 5 h). The resulting extract was chromatographed using a Grace Reveleris flash chromatography system. A C-18 silica gel column was used. A gradient of water/acetonitrile was employed as mobile phase (3 CV from 30% to 70%, 10 CV on 70% and 3 CV from 70% to 100%). Fractions were automatically grouped according to the UV light absorption at 226 and 254 nm. This afforded 3.1 mg of pure sedanolide, whose identity was confirmed via ^1H and ^{13}C NMR, and LC-ESI-MS analysis with literature data comparison (Sbai et al., 2017)



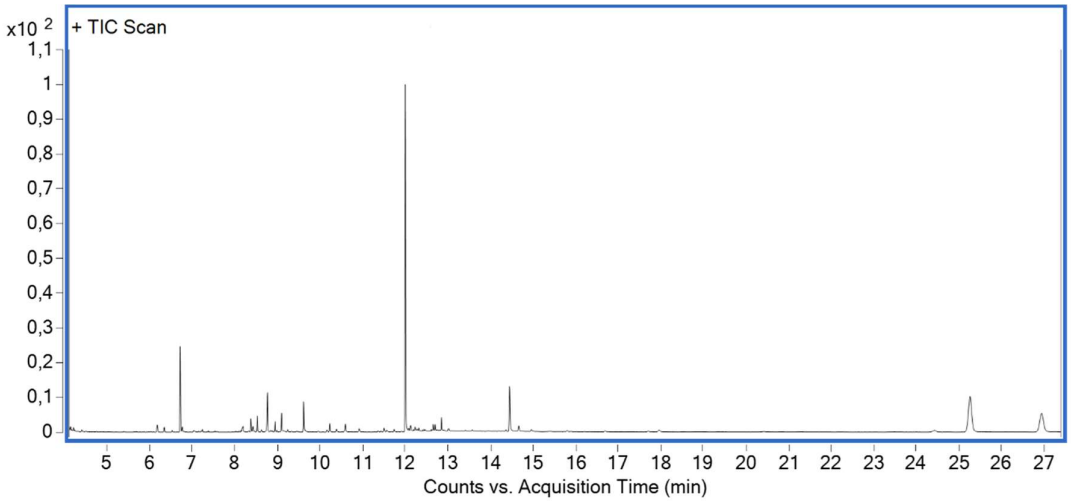
SI Fig.1 - Example of a ^{1}H NMR spectrum of celery waste material water extract (SOX_H), with integration regions employed for the quantification.



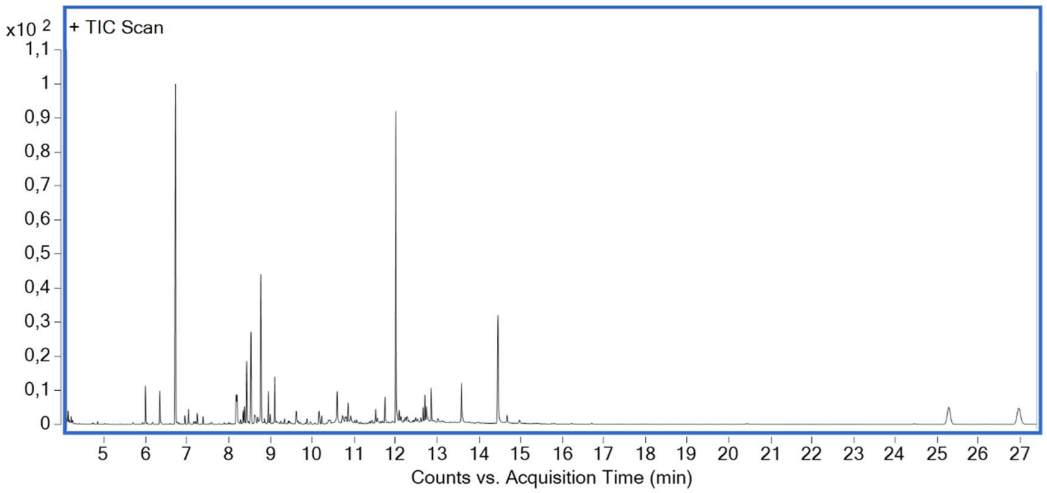
SI Fig. 2 - Illustrative HSQC spectrum of the sample "H2O"

SI Table 1 - Peak list of the illustrative HSQC spectrum of the sample "H2O"

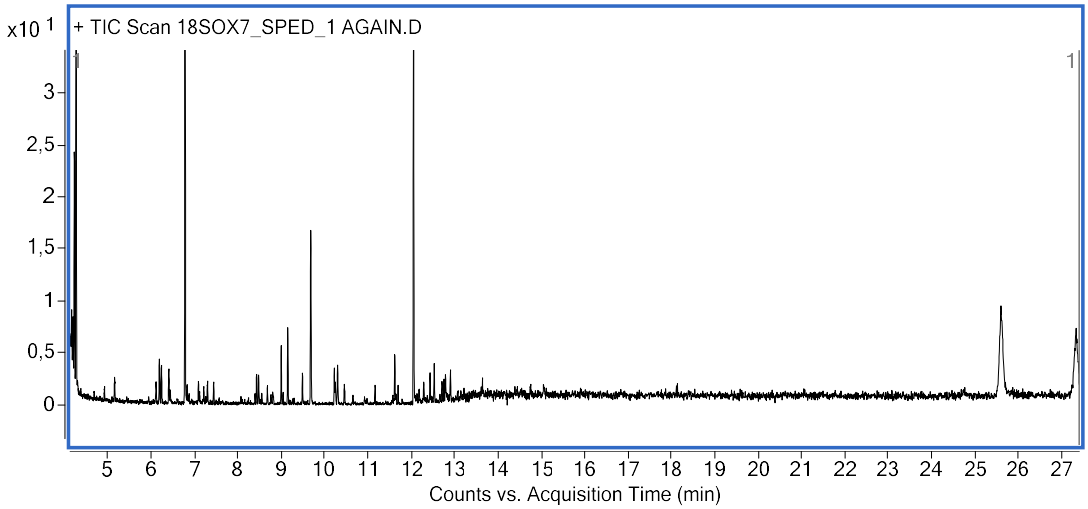
Peak	^{1}H NMR shift (ppm)	^{13}C NMR shift (ppm)
1	3.86	65.84
2	3.78	71.87
3	3.76	73.47
4	3.67	65.84



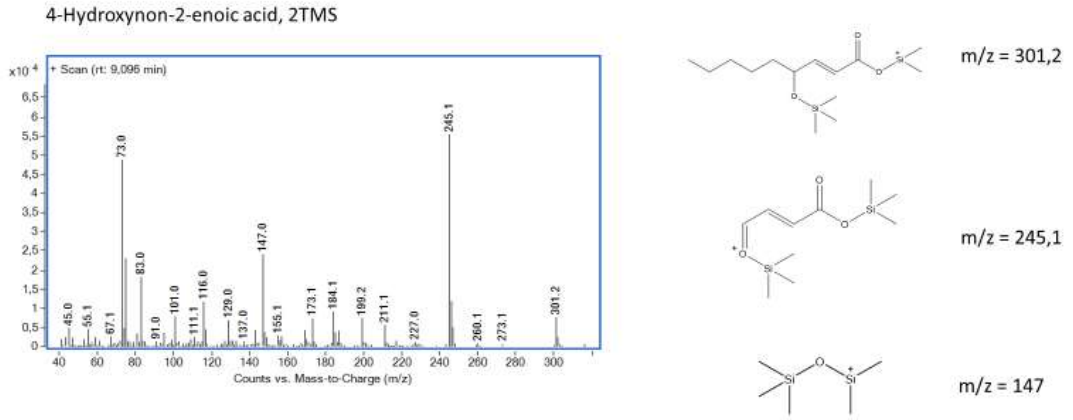
SI Fig. 3 - Total Ion Chromatogram (TIC) of sample SFE1



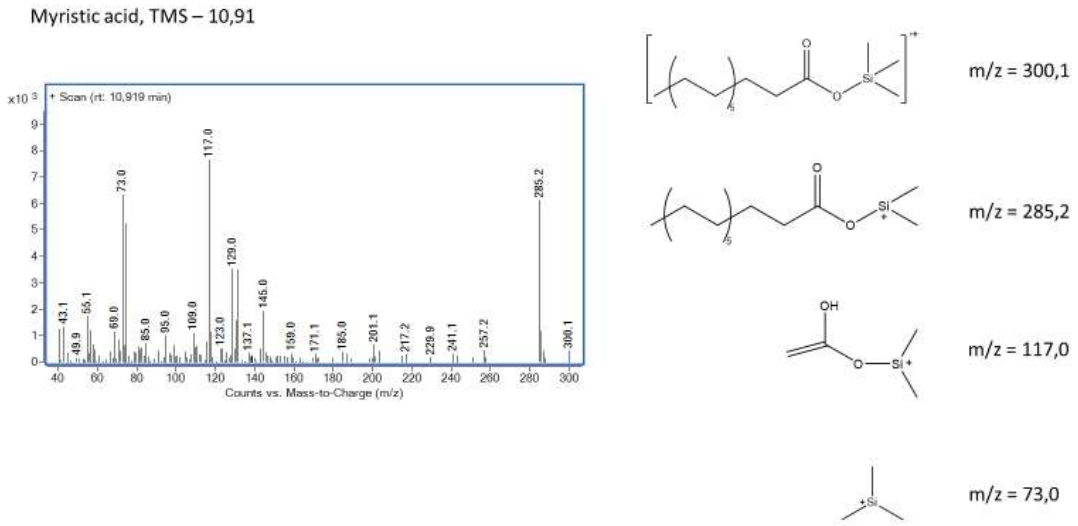
SI Fig. 4 - TIC of sample SFE2



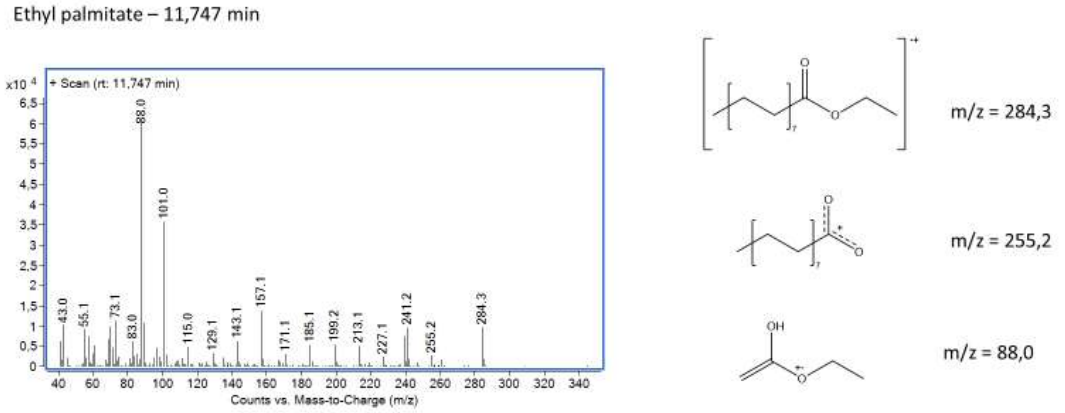
SI Fig. 5 - TIC of sample SOX



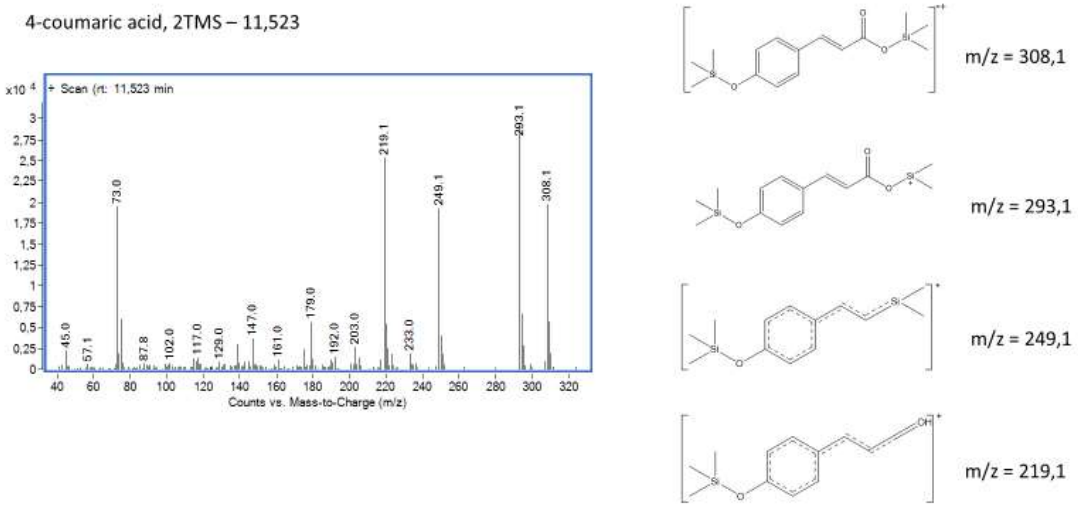
SI Fig. 6- Mass spectrum of 4-hydroxynon-2-enoic acid with assignments of main identifying fragments



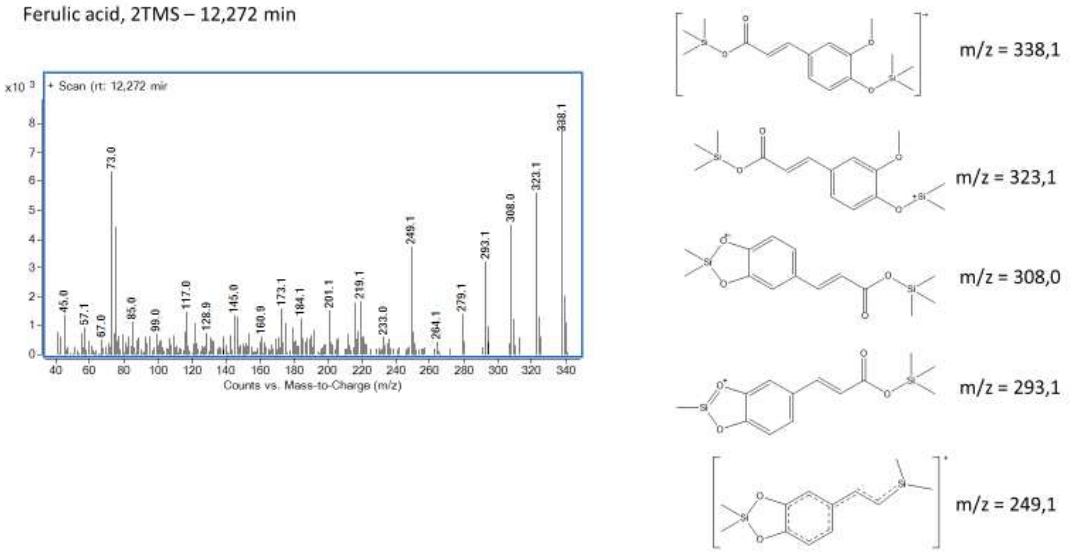
SI Fig. 7 - Mass spectrum of myristic acid with assignments of main identifying fragments



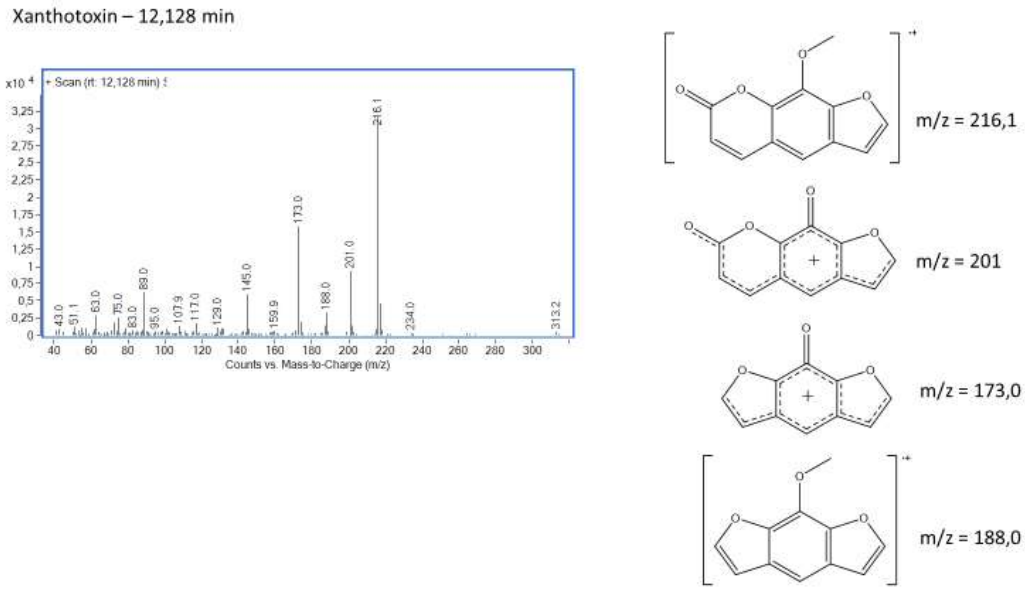
SI Fig. 8 - Mass spectrum of ethyl palmitate with assignments of main identifying fragments



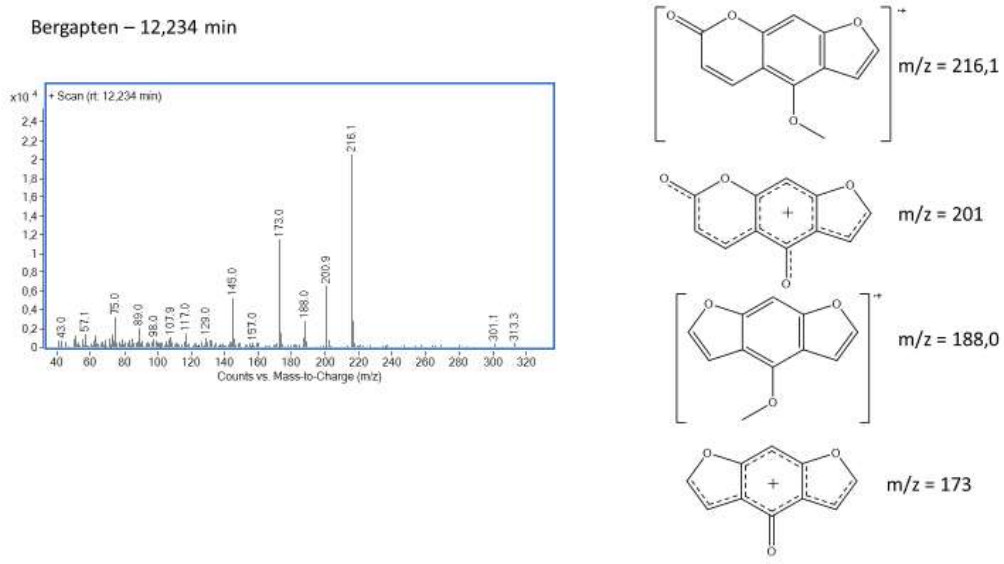
SI Fig. 9 - Mass spectrum of 4-coumaric acid, 2TMS with assignments of main identifying fragments



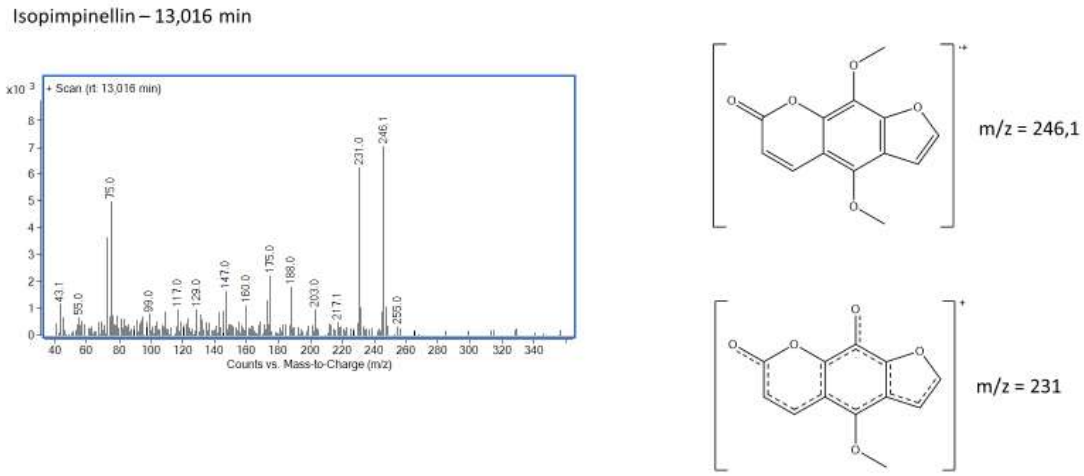
SI Fig. 10 - Mass spectrum of ferulic acid, 2TMS with assignments of main identifying fragments



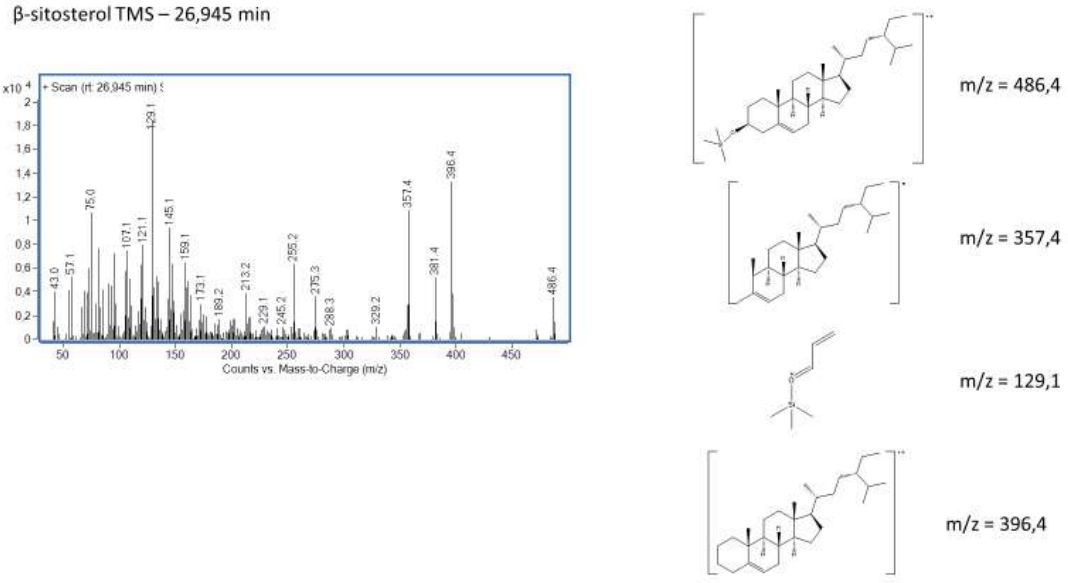
SI Fig. 11 - Mass spectrum of xanthotoxin with assignments of main identifying fragments



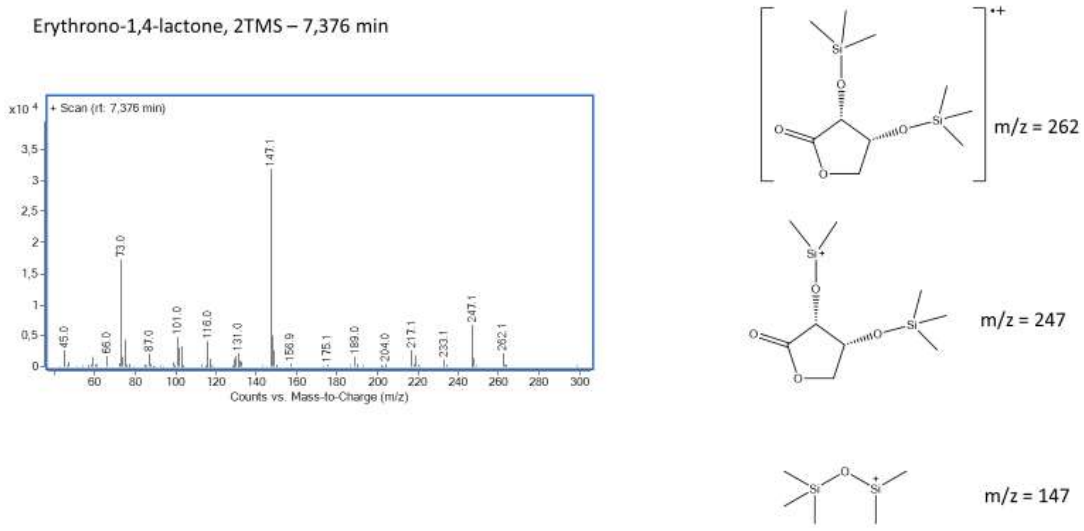
SI Fig. 12 - Mass spectrum of bergapten with assignments of main identifying fragments



SI Fig. 13 - Mass spectrum of isopimpinellin with assignments of main identifying fragments



SI Fig. 14 - Mass spectrum of β-sitosterol TMS with assignments of main identifying fragments



SI Fig. 15 - Mass spectrum of erythrono-1,4-lactone TMS with assignments of main identifying fragments